

CHARACTERIZATION OF ADENOSINE RECEPTOR(S) INVOLVED IN  
ADENOSINE-INDUCED BRONCHOCONSTRICTION IN  
AN ALLERGIC MOUSE MODEL

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Adenosine Receptors and Bronchoconstriction

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## ABSTRACT

We recently reported that adenosine caused bronchoconstriction and enhanced airway inflammation in an allergic mouse model. In this study, we further report the characterization of the subtype of adenosine receptor(s) involved in bronchoconstriction. NECA, a non-selective adenosine agonist, elicited bronchoconstriction in a dose-dependent manner. Little effects of CPA ( $A_1$ -selective agonist) and CGS21680 ( $A_{2A}$ -selective agonist) compared to NECA were observed in this model. CL-IB-MECA, an  $A_3$ -selective receptor agonist, produced a dose-dependent bronchoconstrictor response, which was blocked by selective  $A_3$  antagonist, MRS1523. However, MRS1523 only partially inhibited NECA-induced bronchoconstriction. Neither selective  $A_1$  nor  $A_{2A}$  antagonists affected NECA-induced bronchoconstriction. Enprofylline, a relatively selective  $A_{2B}$  receptor antagonist, blocked partly NECA-induced bronchoconstriction. Furthermore, a combination of enprofylline and MRS1523 completely abolished NECA-induced bronchoconstrictor response. Using RT-PCR, we found that all four adenosine receptor subtypes are expressed in control lungs. Allergen sensitization and challenge significantly increased transcript levels of the  $A_{2B}$  and  $A_3$  receptors whereas the  $A_1$  receptor message decreased. No change in transcript levels of  $A_{2A}$  receptors was observed after allergen sensitization and challenge. These findings suggested that  $A_{2B}$  and  $A_3$  adenosine receptors play an important role in adenosine-induced bronchoconstriction in our allergic mouse model. Finally, whether the airway effects of the receptor agonists/antagonists are direct or indirect need further investigations.

Key Words: adenosine agonists, adenosine antagonists, mouse lung, asthma

## INTRODUCTION

Among the many actions of adenosine, several lines of evidence suggest a contribution of adenosine to the pathophysiology of asthma. Adenosine is present in higher concentrations in the bronchoalveolar lavage fluid (BALF) of asthmatics (8). Airway preparations from allergic asthmatic subjects are more sensitive to the contractile responses of adenosine and related analogues (4). Inhaled adenosine causes bronchoconstriction in asthmatics and allergic non-asthmatics compared with normal subjects (24).

The response of the asthmatic airways to adenosine involves the activation of extracellular adenosine receptor(s). Theophylline, a non-selective adenosine receptor antagonist, selectively (relative to histamine) inhibited adenosine-induced bronchoconstriction at doses generating plasma concentrations that are insufficient to inhibit phosphodiesterases but well above those needed to block adenosine receptors (22). Moreover, both rebound hyperresponsiveness to adenosine and exacerbation of symptoms have been reported when chronic theophylline therapy was withdrawn (36).

Adenosine receptors are seven-transmembrane-spanning receptors that are coupled to effector system through heterotrimeric G proteins. Four subtypes of adenosine receptors, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, have been identified based on the order of agonist potency and antagonist affinity, G-protein coupling, cellular responses and receptor cloning studies (11). Each of these receptor subtypes is expressed in lung and may have a role in asthma. Most evidence indicates that activation of the A<sub>1</sub> receptors, and to some extent the A<sub>3</sub> receptors, induces adenylyl cyclase inhibition with subsequent decrease in adenosine 3':

5'-cyclic monophosphate (cAMP) level. This intracellular signal induces contraction of the airway smooth muscle. In contrast, when adenosine activates the  $A_{2A}$  receptors, cAMP level increases which causes airway relaxation (29). Adenosine  $A_{2B}$  receptors have long been known to be coupled to  $G_s$  protein, which mediate the activation of adenylyl cyclase (12,33). Recent studies indicate that  $A_{2B}$  receptors are also coupled to  $G_q$  and result in  $Ca^{2+}$  mobilization and mitogen-activated protein kinase (MAPK) activation (20,21). Also, the  $A_3$  receptors have been suggested to mediate the facilitation of antigen-induced release of bronchoconstrictor mediators through inositol triphosphate ( $IP_3$ ) and  $Ca^{2+}$  pathway (34). Despite the pivotal role that adenosine receptors play in bronchoconstriction and inflammatory reactions under allergic conditions, the subtype(s) of adenosine receptors mediating bronchoconstriction remain poorly defined. The accumulated evidences show that the receptor subtype varies widely among species. For example, the involvement of  $A_3$  receptors in guinea-pig (18),  $A_1$  receptors on airway smooth muscle cell in rabbit (28), and a combination of  $A_1$ ,  $A_{2B}$ , and  $A_3$  receptors in BDE rat (31). In mice, our data (9), and others (6) showed that exogenous and endogenous adenosine mediate bronchoconstriction and airway hyperresponsiveness through specific adenosine receptors, but the relative contribution of each adenosine receptor subtype mediating bronchoconstriction in mice is still unresolved.

The present study was initiated following our previous study demonstrating that ragweed sensitization and challenge of mice induced a profound increase in bronchoconstrictor response to adenosine and lung inflammation (9). Here, we investigated the involvement of the subtype of adenosine receptor(s) mediating bronchoconstriction to adenosine using selective adenosine receptor agonists and

antagonists, and studied a profile of adenosine receptor expression in allergic mouse lung using RT-PCR.

## MATERIALS AND METHODS

### Mice and Sensitization

Male BALB/c mice, 6 to 8 wk 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 a method described previously (9). Briefly, mice were sensitized with two i.p. injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 µg per dose with 200µl Imject<sup>®</sup> Alum (Pierce Laboratories, Rockford, IL) on days 1 and 6. Non-sensitized control animals only received the Imject<sup>®</sup> alum with the same volumes. 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 minutes both in the morning and afternoon on days 11, 12 and 13.

### Experimental Protocol

**Airway responsiveness to adenosine agonists:** Airway responsiveness was assessed with whole-body plethysmograph (WBP, Buxco Max II, Troy NY). This system estimates total pulmonary airflow using a dimensionless parameter known as enhanced pause (Penh). Pressure differences were used to extrapolate Penh value, which is a function of the sum of the airflows in the upper and lower respiratory tracts during a respiratory cycle. Penh has been shown to correlate with direct measurement of airway

obstruction ( $R_L$  and  $C_{dyn}$ ) (17). 24 hours after the last challenge with aerosolized ragweed or saline, mice were placed in the chamber, and exposed to adenosine agonists 5'-N-ethylcarboxamidoadenosine (NECA, a non-selective adenosine receptor agonist), N<sup>6</sup>-cyclopentyladenosine (CPA, a selective A<sub>1</sub> adenosine receptor agonist) in 10% ethanol, CGS21680 (a selective A<sub>2A</sub> adenosine receptor agonist) in 0.9% saline, CL-IB-MECA (a selective A<sub>3</sub> adenosine receptor agonist) in 20% DMSO with Buxco Aerosol Delivery System (Version 1.5. Sharon, CT) at 2.5 L/min of the dilution flow and 0.15 L/min of the trickle flow for 2 min at increasing concentrations (11.72 – 375 µg/ml) for dose-responsiveness. The next dose was not given until the animal returned to baseline Penh. Airway responsiveness was expressed as percentage increase in Penh compared to vehicle. Readings were taken for 5 minutes following each nebulization. For the quantification of the dose-responsiveness to adenosine agonists, the linear regression of Penh was calculated for individual mice. The dose corresponding to an increase in Penh of 100% (EC<sub>100</sub>) was determined and average dose in different groups was compared by analysis of variance.

**Adenosine antagonist challenges:** Adenosine antagonists, DPCPX (A<sub>1</sub> selective, 10<sup>-4</sup> M), SCH58261 (A<sub>2A</sub> selective, 10<sup>-4</sup> M), 3-n-propylxanthine (enprofylline, a relative A<sub>2B</sub> antagonist, 10<sup>-4</sup> M) in 10% ethanol, MRS1523 (A<sub>3</sub> selective, 10<sup>-4</sup> M) in 20% DMSO was aerosolized with Buxco Aerosol Delivery System (Version 1.5. Sharon, CT) at 2.5 L/min of the dilution flow and 0.15 L/min of the trickle flow for 5 minutes. After 15 minutes, dose-responsiveness to a corresponding adenosine agonist and NECA were performed in the same manner as mentioned above. Pulmonary measurements were recorded before and after pretreatment with an adenosine antagonist to determine its effect on basal

bronchial tone. Also, effect of vehicle *per se* on Penh was examined.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):** Mice were immunized with the protocol described in the earlier section. 24 hours after the last challenge with aerosolized allergen or 0.9% saline, mice were killed by i.p. injection (0.1 ml pentobarbitone sodium 200mg/ml). Lungs were taken and immediately frozen in liquid nitrogen. Total RNA was isolated from the mouse lung of both ragweed sensitized and challenged mice and control animals with TRIzol Reagent (Roche Diagnostics Corp. Indianapolis, IN) according to the manufacturer's instructions, and RT-PCR was conducted as described earlier (5). Briefly, for the RT reaction, 5 µg of purified total RNA was reverse transcribed in the presence of an anchored oligo-p (dT)<sub>15</sub> primer, by use of AMV reverse transcriptase (Roche Diagnostics Corp. Indianapolis, IN) based on manufacturer's recommendations. Sets of specific primers for adenosine receptors for A<sub>1</sub> (37), A<sub>2A</sub> (15), A<sub>2B</sub> (5), and A<sub>3</sub> (3) are presented in Table 1.

Single stranded cDNA products were denatured and subjected to PCR amplification (35 cycles). Each PCR cycle consisted of denaturing at 94 °C for 5 min (for A<sub>1</sub> and A<sub>2B</sub> receptors), and at 94 °C for 3 min (for A<sub>2A</sub> and A<sub>3</sub> receptors); annealing at 60 °C for 1 min (for A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors), and at 65 °C for 30 seconds (for A<sub>1</sub> receptors); extension at 72 °C for 2 min (for A<sub>1</sub> and A<sub>2B</sub> receptors), and at 72 °C for 1 min (for A<sub>2A</sub> and A<sub>3</sub> receptors). A final extension of 72 °C for 7 min (for A<sub>1</sub> and A<sub>2B</sub> receptors), and of 72 °C for 10 min (for A<sub>2A</sub> and A<sub>3</sub> receptors) was added after the 35<sup>th</sup> amplification cycle. The reaction mixture contained: 0.2 mM dNTP, 2.5 µ Taq DNA polymerase (Roche Diagnostics Corp. Indianapolis, IN), 1×PCR buffer (1.5 mM Mg<sup>2+</sup>) with GeneAmp PCR system (model 9600, Perkin-Elmer, USA). Amplification of β-actin mRNA served as

control. The PCR products were separated on 2.5% agarose gels with ethidium bromide. The resulting data were analyzed using FlourChem™ digital Imagine System with AlphaEaseFC™ software (Alpha Innotech Corporation, CA). The relative expression level of adenosine receptor mRNA was calculated in comparison with  $\beta$ -actin. Results are expressed as the mean  $\pm$  SE.

### Chemicals

Ragweed pollen extract was purchased from Greer Laboratories (Lenoir, NC). Imject® Alum was purchased from Pierce Laboratories (Rockford, IL). N<sup>6</sup>-Cyclopentyladenosine (CPA), 2-p- (2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680), 5'-N-Ethylcarboxamidoadenosine (NECA), 1,3, -dipropyl-8-cyclopentylxathine (DPCPX), 3-n-propylxanthine (enprofylline), 2,3-diethyl-4, 5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS1523), ethanol and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine (SCH58261) was a gift from Dr. A. Monopoli (Shearing Plough; Milan, Italy). 2-chloro-N<sup>6</sup>- (3-iodobenzyl)-9-[5-(methylcarbamoyl)- $\beta$ -D-ribofuranosyl]-adenosine (CL-IB-MECA) was obtained by SRI International from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program.

### Statistical Analysis

Experimental values are presented as mean  $\pm$  SE. EC<sub>100</sub> (the dose of an agonist required producing a 100% increase in Penh) was calculated. Analysis of variance was

used to determine the level of significance among all groups. Pairs of groups were compared with unpaired two-tailed Student's *t*-test. The *p* value for significance was set at 0.05.

## RESULTS

**Dose-responsiveness to adenosine receptor agonists and the effect of corresponding adenosine receptor antagonists:** Aerosol challenge with NECA, a non-selective adenosine receptor agonist, elicited a dose-dependent increase in Penh in both ragweed sensitized and challenged and control mice. Bronchoconstrictor responsiveness to NECA was significantly enhanced in allergen sensitized and challenged mice compared to the controls. The maximum responsiveness to NECA (375 µg/ml) was  $405.9 \pm 35.6\%$  in sensitized and challenged mice and  $181.8 \pm 7.7\%$  in controls, respectively ( $p < 0.001$ ). Dose of NECA required to produce a 100% increase in Penh ( $EC_{100}$ ) was: 30.66 µg/ml (95% confidence limits: 24.75 to 36.58) in ragweed sensitized and challenged mice, and 142.03 µg/ml (95% confidence limits: 124.47 to 159.60) in control animals, respectively ( $P < 0.001$ , Figure 1). The baseline Penh values between control ( $0.72 \pm 0.06$ ) and ragweed sensitized and challenged mice ( $0.78 \pm 0.05$ ) were not different. Vehicle (10% ethanol aerosolized for 2 min) used in experiment with agonists had no significant effect on Penh in both control and immunized mice. Also, 10% ethanol aerosolized for 5 min (vehicle for antagonists) was without an effect on basal bronchial tone.

Since NECA, like adenosine has affinity for each of the four-adenosine receptor subtypes (19), the question arises as to which adenosine receptor subtype is being activated in NECA-induced bronchoconstriction. To distinguish the subtype of adenosine receptors involved in NECA-induced bronchoconstrictor responsiveness, selective adenosine agonists and antagonists were employed. CPA is a potent and selective agonist at  $A_1$  receptors with reported inhibitor constant ( $K_i$ ) values of 0.59, 460 and 240 nM at rat

A<sub>1</sub>, A<sub>2A</sub> and A<sub>3</sub> receptors, respectively (11). Aerosol challenge of mice with CPA elicited little change in Penh in both control and immunized mice (Figure 2A). Further experimentation was done to examine the bronchoconstrictor responsiveness to NECA with a selective A<sub>1</sub> receptor antagonist DPCPX, for which the K<sub>i</sub> values at rat A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors are 0.3 nM, 340 nM, and >10μM, respectively (11). There was no effect of DPCPX on dose-responsiveness to NECA observed after mice pretreated with DPCPX (Figure 2B). DPCPX *per se* had no effect on baseline Penh in both groups. Penh values before and after DPCPX under baseline condition were (0.68±0.03) and (0.62±0.05) in control animals, and (0.73±0.06) and (0.67±0.08) in sensitized and challenged mice, respectively, (*p*>0.05).

CGS21680 is the most potent, highly selective agonist at A<sub>2A</sub> receptors and is the ligand of choice for the characterization of A<sub>2A</sub> receptors. Aerosol challenge with CGS21680 did not affect the Penh (Figure 3A). Pretreatment with an A<sub>2A</sub> receptor antagonist, SCH58261, a potent and highly selective A<sub>2A</sub> receptor antagonist both *in vivo* and *in vitro* (30) was unable to alter the bronchoconstrictor dose-response curve to NECA in either control or sensitized and challenged groups (Figure 3B). There was no difference in baseline Penh value before and after SCH58261 in both groups of animals. The values were 0.64±0.04 and 0.66±0.05 (before vs after SCH58261) in control group, and 0.74±0.04 and 0.79±0.05 (before vs after SCH58261) in sensitized and challenged group, respectively.

CL-IB-MECA is a highly potent and selective A<sub>3</sub> receptor agonist with K<sub>i</sub> values of 820, 470, and 0.33 nM at rat A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors, respectively (11). CL-IB-MECA challenge produced marked bronchoconstrictor responsiveness in a dose-dependent

manner (Figure 4A). The maximum responsiveness to CL-IB-MECA (375  $\mu\text{g/ml}$ ) was  $334.2\pm 34.6\%$  in sensitized and challenged mice and  $169\pm 19.4\%$  in controls, respectively ( $p<0.001$ ). The dose of CL-IB-MECA required to produce a 100% increase in Penh ( $\text{EC}_{100}$ ) was: 62.81  $\mu\text{g/ml}$  (95% confidence limits: 52.52 to 73.10) in ragweed sensitized and challenged mice, and 158.55  $\mu\text{g/ml}$  (95% confidence limits: 121.75 to 195.35) in control animals, respectively. CL-IB-MECA-induced bronchoconstrictor responsiveness was completely inhibited by an  $A_3$ -selective antagonist, MRS1523 that is effective in various species (11) (Figure 4A). MRS1523 itself did not affect basal bronchial tone. The basal Penh values before and after pretreatment with MRS1523 were:  $0.61\pm 0.03$  and  $0.72\pm 0.09$  in control mice ( $P>0.05$ ), and in sensitized and challenged animals were  $0.71\pm 0.05$  and  $0.64\pm 0.07$  ( $P>0.05$ ), respectively. Vehicle alone (20% DMSO aerosolized for 2 min or 5 min) had no effect on Penh in both control and allergen sensitized and challenged mice.

Furthermore, the effect of MRS1523 on a dose-response curve for NECA was also tested. It was found that NECA-induced bronchoconstriction was partially inhibited by MRS1523 (Figure 4B), especially at the highest concentration (375  $\mu\text{g/ml}$  of NECA). This suggests the involvement of other adenosine receptors, possibly the  $A_{2B}$  receptors, in addition to  $A_3$  receptors.

Since there is a lack of availability of  $A_{2B}$ -selective antagonists commercially, enprofylline, which is somewhat selective for  $A_{2B}$  adenosine receptors with  $K_i$  values of 156, 32, 7, and 65 $\mu\text{M}$  at human  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  receptors, respectively (10), was used to examine the effect of  $A_{2B}$  receptors in NECA-induced bronchoconstrictor responsiveness. As expected, enprofylline did block NECA-induced bronchoconstriction

as reflected by rightward shift of the dose-response curve (Figure 5). EC<sub>100</sub> changed from 30.66 µg/ml (95% confidence limits: 24.75 to 36.58) in sensitized and challenged mice to 157.40 µg/ml (95% confidence limits: 114.59 to 200.21) after pretreatment with enprofylline in sensitized and challenged mice ( $p<0.01$ ). Also, a combination of enprofylline and MRS1523 completely inhibited the bronchoconstrictor responsiveness to NECA in both groups (Figure 6), which was different from the effect of MRS1523 alone on a dose-response curve for NECA (Figure 4B), suggesting the involvement of A<sub>2B</sub> and A<sub>3</sub> receptors in NECA-induced bronchoconstriction.

**Adenosine receptor transcript levels in the mouse lungs:** Adenosine receptor transcript levels were quantified in total RNA extracts isolated from whole lungs of both ragweed sensitized and challenged mice and control animals. RT-PCR revealed expression of four subtypes of adenosine receptors in the control lungs, with relatively high levels of A<sub>2B</sub> receptor message, intermediate levels of A<sub>1</sub> and A<sub>3</sub> receptor message, and low levels of A<sub>2A</sub> receptors (Figure 7A). Mice sensitized and challenged with ragweed showed changes in the message for adenosine receptors in lungs. There were significant elevations in transcript levels of A<sub>2B</sub> and A<sub>3</sub> receptors after ragweed sensitization and challenge, which increased by 42.58% for A<sub>2B</sub> receptors ( $p<0.01$ ) and 29.05% for A<sub>3</sub> receptors ( $p<0.05$ ), respectively, and a decrease in the expression of A<sub>1</sub> adenosine receptors by 22.85% ( $p<0.05$ ). Little change in the expression of A<sub>2A</sub> receptors was observed (-4.39%) with a  $p$  value  $>0.05$  (Figure 7B).

## DISCUSSION

Consistent with our previous study (9), our present results show that NECA, a non-selective adenosine receptor agonist causes bronchoconstriction, which is significantly greater in allergen systemically sensitized, airway challenged mice compared to control animals. Furthermore, the A<sub>1</sub> agonist CPA and A<sub>2A</sub> agonist CGS21680 produced little increase in Penh in this allergic mouse model. The selective A<sub>1</sub> or A<sub>2A</sub> antagonist did not affect bronchoconstrictor responsiveness to NECA.

However, the A<sub>3</sub> agonist, CL-IB-MECA induced bronchoconstriction in a dose-dependent manner, which was completely inhibited by the selective A<sub>3</sub> antagonist, MRS1523 (Figure 4A). At the same time, NECA-induced bronchoconstriction was partially inhibited by MRS1523, especially at the highest concentration of NECA, indicating the involvement of other adenosine receptors, possibly A<sub>2B</sub> receptors. To confirm our observations further, a relatively selective A<sub>2B</sub> receptor antagonist, enprofylline, was employed to study the effect of A<sub>2B</sub> receptors on NECA-induced bronchoconstriction. Enprofylline showed an attenuation of NECA-induced bronchoconstriction (Figure 5). As enprofylline is a relatively selective A<sub>2B</sub> antagonist with *K<sub>i</sub>* values of 7 μM and 65 μM at A<sub>2B</sub> and A<sub>3</sub> receptors, respectively (10), it was still difficult to conclude the role of A<sub>2B</sub> receptors from this experiment. Further supporting data were obtained using a combination of enprofylline with MRS1523, which showed that the partial inhibition of NECA-induced bronchoconstriction was completely blocked by a combination of enprofylline and MRS1523. These results were supported by the transcript levels of both A<sub>2B</sub> and A<sub>3</sub> receptors being higher in the sensitized and challenged mice as opposed to control mice. Taken together, these results suggested that

A<sub>2B</sub> and A<sub>3</sub> adenosine receptors are possibly involved in NECA-induced bronchoconstriction in an allergic mouse model.

Adenosine and its agonists-induced bronchoconstriction have been studied in several animal models. A series of studies carried out by Pauwels and colleagues (31,32) showed that the BDE rats respond to adenosine agonists with an order of potency: NECA=CPA>N<sup>6</sup>-2-(4-aminophenyl) ethyladenosine (APNEA)>N<sup>6</sup>-cyclohexyladenosine (CHA)>R-PIA>CGS21680. In the same study, A<sub>3</sub>-selective agonist produced a dose-dependent bronchoconstriction, and selective A<sub>1</sub> receptor antagonists KF15372 and KW3902 inhibited bronchoconstrictor responsiveness to NECA (31,32). These observations led the investigators to conclude that adenosine-induced bronchoconstriction in BDE rat was most likely through A<sub>1</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors (31,32). In Brown Norway rats, Hannon and coworkers (13) suggested the involvement of A<sub>2B</sub> receptors in bronchoconstrictor responsiveness to adenosine. Same investigators, in a more recent study, questioned the role of A<sub>2B</sub> receptors in the bronchoconstrictor responsiveness to adenosine in Brown Norway rats (14). Studies using guinea pig suggested the existence of a mechanism involving adenosine A<sub>3</sub> receptors (18,38). In another animal model, the animals immunized neonatally with ragweed or house dust mite developed bronchoconstrictor responsiveness to adenosine agonists (1). In this model, the order of agonist potency was CPA>>NECA>>CGS, and CPA-induced bronchoconstrictor responsiveness was blocked by a selective A<sub>1</sub> antagonist, DPCPX (2). This pharmacological profile is typical of an adenosine A<sub>1</sub> receptor subtype. In an extension of these studies, Nyce and Metzger (28) confirmed that an aerosolized phosphorothiorate antisense oligodeoxynucleotide directed against the A<sub>1</sub> receptors (but not the appropriate sense control) attenuated

bronchoconstrictor responsiveness of immunized rabbits to either adenosine or adenosine agonists or dust-mite allergen. In mice, to the best of our knowledge, this study, for the first time, demonstrated that adenosine- and its agonist-induced bronchoconstriction was most likely mediated by the activation of A<sub>2B</sub> and A<sub>3</sub> adenosine receptors. Furthermore, an expression of all four subtypes of adenosine receptors was found in normal mouse lung using RT-PCR (Figure 7A). Allergen sensitization and airway challenge produced a significant increase in the transcript levels of A<sub>2B</sub> and A<sub>3</sub> adenosine receptors in mouse lung (Figure 7B).

Whether the effect of receptor activation in this model is a direct effect on airway smooth muscle or secondary to an effect on the activation of other cells (e.g., mast cells, eosinophils, neutrophils, lymphocytes and macrophages) cannot be determined from this study. However, there is evidence that A<sub>2B</sub> adenosine receptors are expressed in human airway smooth muscle cells (26). Adenosine could directly affect airway smooth muscle cell contractility by modulating the [Ca<sup>2+</sup>]<sub>i</sub> through the activation of adenosine A<sub>3</sub> receptors (25). Mice lacking the adenosine A<sub>3</sub> receptors showed the attenuation of adenosine-induced bronchoconstriction (39). The A<sub>1</sub> receptors located directly on the smooth muscle in monkey and rabbit (27,28) since selective depletion of the airway smooth muscle A<sub>1</sub> receptors by antisense reduced the bronchoconstrictor response to adenosine (28). However, our data showed a decrease in transcript level for A<sub>1</sub> receptors after allergen sensitization and challenge of mice as well the absence of CPA-induced bronchoconstriction both in control and sensitized and challenged mice. This discrepancy may be attributed to the different animal model with varying pathophysiological conditions. A decrease in the expression of adenosine A<sub>1</sub> receptors has been reported in

chronic inflammatory disease (16). Diminished A<sub>1</sub> receptor mRNA in mouse lung in our study may reflect a compensatory mechanism for the lung inflammation since pro-inflammatory actions of adenosine A<sub>1</sub> receptor agonists on inflammatory cells have been suggested (7).

A variety of evidence points to the bronchoconstrictor responsiveness to adenosine being a consequence of activation of inflammatory cells in airways, especially mast cells and eosinophils (40,41). It is well documented that A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptor mRNA is expressed in mouse bone marrow-derived mast cells (23,35). In a functional study, Zhong et al reported that enprofylline was able to protect about 30% of the adenosine-dependent mast cell degranulation in ADA-deficient mice (41). These authors further demonstrated with ADA-deficient and A<sub>3</sub> receptor double knockout mice that A<sub>3</sub> receptors also played an important role in adenosine mediated murine lung mast cell degranulation (42). Enhanced expression of adenosine A<sub>3</sub> receptors on eosinophils has also been reported in asthmatic lung (40). Our findings of increased expression of A<sub>2B</sub> and A<sub>3</sub> adenosine receptors in ragweed sensitized and challenged mouse lung was also confirmed by Chunn and colleagues in partially ADA-deficient mouse lung (6).

In summary, our data show an increased expression of A<sub>2B</sub> and A<sub>3</sub> receptor transcripts, and no change in A<sub>2A</sub> receptor transcript with a decrease in A<sub>1</sub> message in sensitized and challenged mice. Although A<sub>1</sub>/A<sub>2A</sub> receptors were expressed in mouse lung, based on Penh they seemed to play a little role in adenosine-mediated bronchoconstriction in our studies. Taking these data together, we conclude that adenosine A<sub>2B</sub> and A<sub>3</sub> receptors play an important role in adenosine- and its agonist-induced bronchoconstriction in our allergic mouse model.

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Table 1. PCR primers and PCR product sizes

Primer	Nucleotides	Sequence	Product sizes
A <sub>1</sub> Forward	295-314	5'-TGT CCT CAT CCT CAC CCA GA-3'	
A <sub>1</sub> Reverse	586-605	5'-GCA CCC ACA CGA AGA AGT TG-3'	310
A <sub>2A</sub> Forward	1488-1511	5'-GGT CAG CCT CCG TCT CAA CGG CCA-3'	
A <sub>2A</sub> Reverse	1705-1728	5'-TCA GGA CAC TCC TGC TCC ATC CTG-3'	241
A <sub>2B</sub> Forward	699-723	5'-GCC TCG AGT GCT TTA CAG ACC CCC-3'	
A <sub>2B</sub> Reverse	848-873	5'-GAA AGT TGA CTG TCC CCC GGC CTG-3'	175
A <sub>3</sub> Forward	132-152	5'-GAC CAC CAG CTT CTA TTT CA-3'	
A <sub>3</sub> Reverse	660-680	5'-GTC TTG AAC TCC CGA /TCC-3'	350

Figure legends:

Figure 1. Dose-response to NECA. CON: control group; SEN: sensitized group. n=8-10 for each group. Values are means  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

Figure 2. A: Dose-response to CPA. B: Dose-response to NECA without and with pretreatment with DPCPX ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+DPCPX: control mice pretreated with DPCPX; SEN+DPCPX: sensitized mice pretreated with DPCPX. n=8-10 for each group. Values are mean  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

Figure 3. A: Dose-response to CGS21680. B: Dose-response to NECA without and with pretreatment with SCH58261 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+SCH58261: control mice pretreated with SCH58261; SEN+ SCH58261: sensitized mice pretreated with SCH58261. n=8-10 for each group. Values are mean  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

Figure 4. A: Dose-response to CL-IB-MECA without and with pretreatment with MRS1523 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+MRS1523: control mice pretreated with MRS1523; SEN+MRS1523: sensitized mice pretreated with MRS1523. B: Dose-response to NECA without and with pretreatment with MRS1523 ( $10^{-4}$  M). n=8-10 for each group. Values are mean  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

Figure 5. Dose-response to NECA without and with pretreatment with enprofylline ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+Enprofylline: control mice pretreated with enprofylline; SEN+Enprofylline: sensitized mice pretreated with enprofylline. n=8-10 for each group. Values are means  $\pm$  SE. \*  $p < 0.05$ , compared with CON group, #  $p < 0.05$ , compared with CON+Enprofylline group.

Figure 6. Dose-response to NECA without and with the pretreatment with Enprofylline ( $10^{-4}$  M) plus MRS1523 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+Enprofylline/MRS1523: control mice pretreated with enprofylline plus MRS1523; SEN+Enprofylline/MRS1523: sensitized mice pretreated with enprofylline plus MRS1523. n=8-10 for each group. Values are means  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

Figure 7. A: Transcription level for adenosine receptors in control mouse lung. B: Percentage changes in transcription level for adenosine receptors in lungs after allergen sensitization and challenge of mice. n=12-19 for each subtype of adenosine receptors. Values are mean  $\pm$  SE. \*  $p < 0.05$ , compared with control mice.

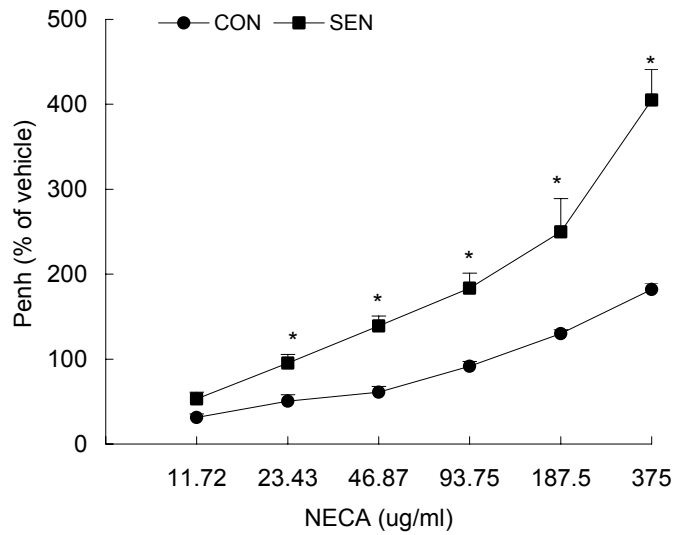


Figure 1. Dose-response to NECA. CON: control group; SEN: sensitized group. n=8-10 for each group. Values are means +/- SE. \* p<0.05, compared with CON group.

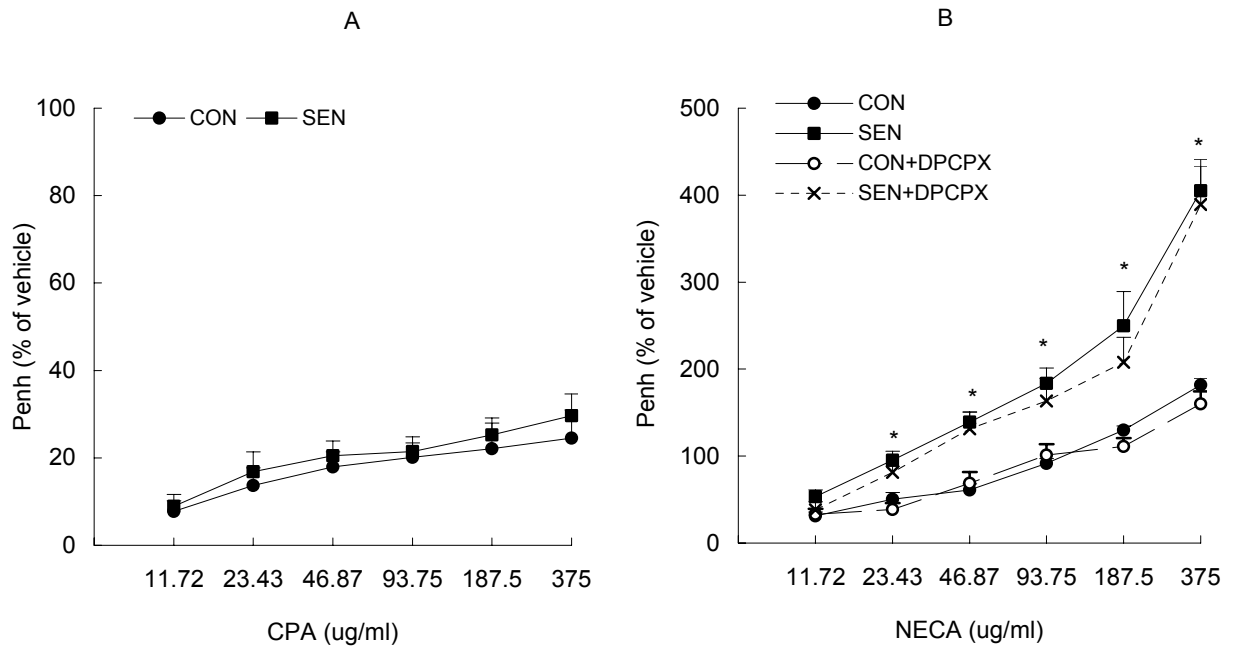


Figure 2. A: Dose-response to CPA. B: Dose-response to NECA without and with pretreatment with DPCPX ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+DPCPX: control mice pretreated with DPCPX; SEN+DPCPX: sensitized mice pretreated with DPCPX. n=8-10 for each group. Values are mean $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

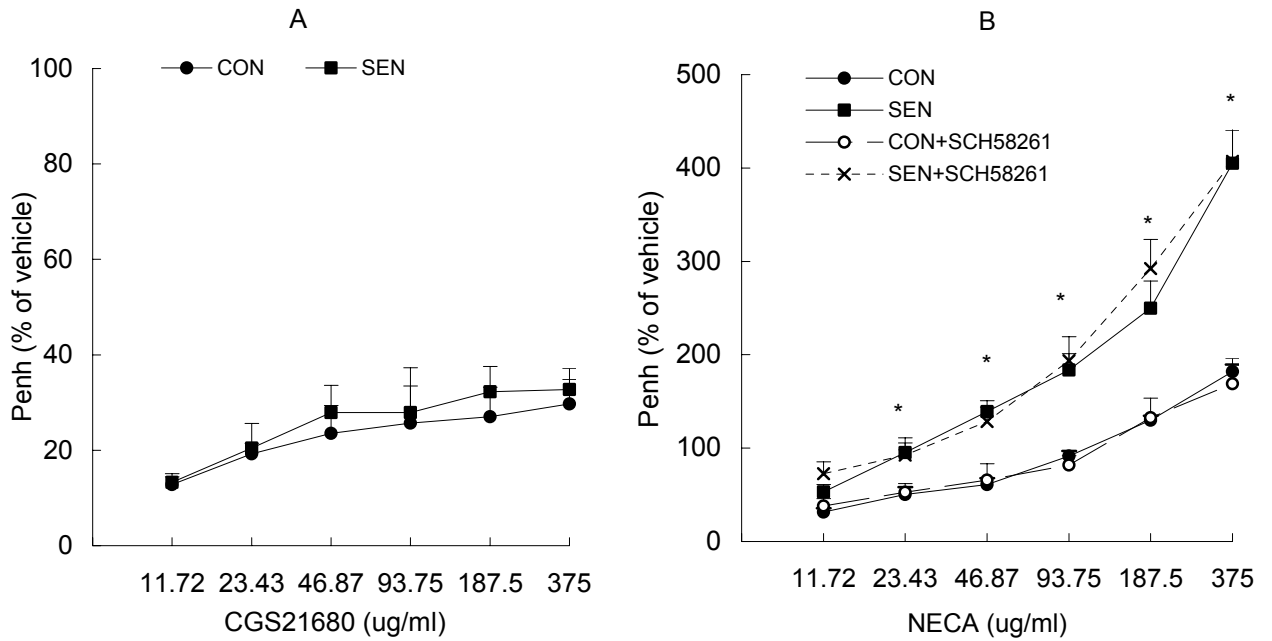


Figure 3. A: Dose-response to CGS21680. B: Dose-response to NECA without and with pretreatment with SCH58261 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+SCH58261: control mice pretreated with SCH58261; SEN+ SCH58261: sensitized mice pretreated with SCH58261. n=8-10 for each group. Values are mean $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

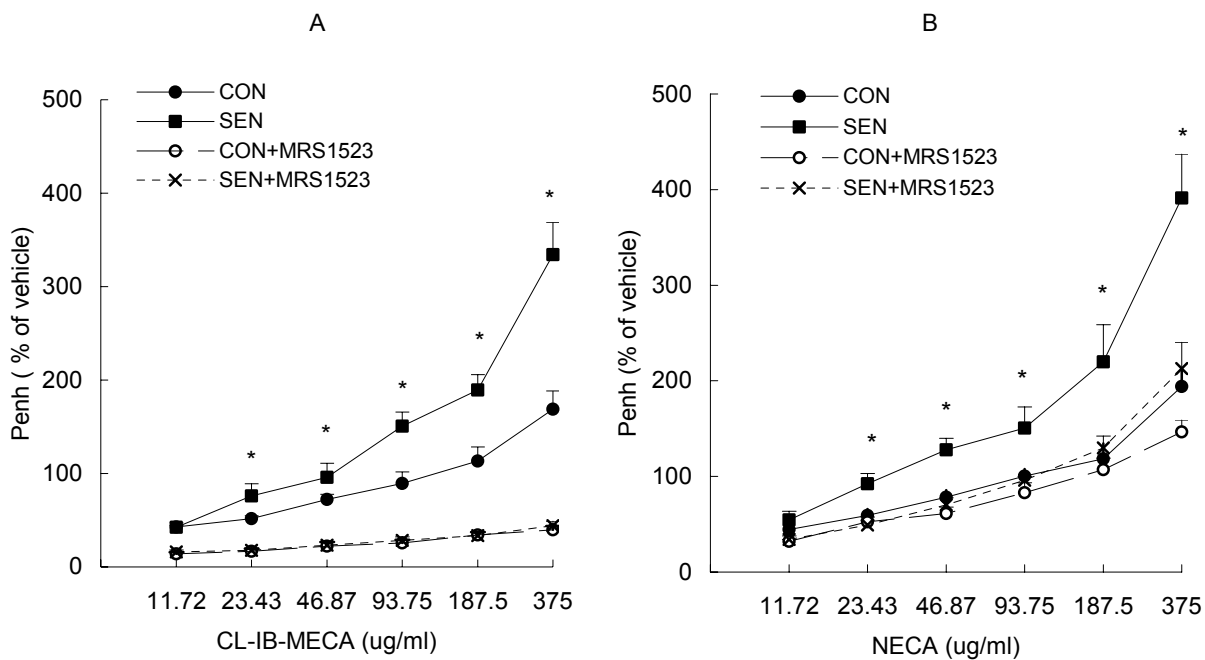


Figure 4. A: Dose-response to CL-IB-MECA without and with pretreatment with MRS1523 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+MRS1523: control mice pretreated with MRS1523; SEN+MRS1523: sensitized mice pretreated with MRS1523. B: Dose-response to NECA without and with pretreatment with MRS1523 ( $10^{-4}$  M). n=8-10 for each group. Values are mean $\pm$  SE. \* p<0.05, compared with CON group.

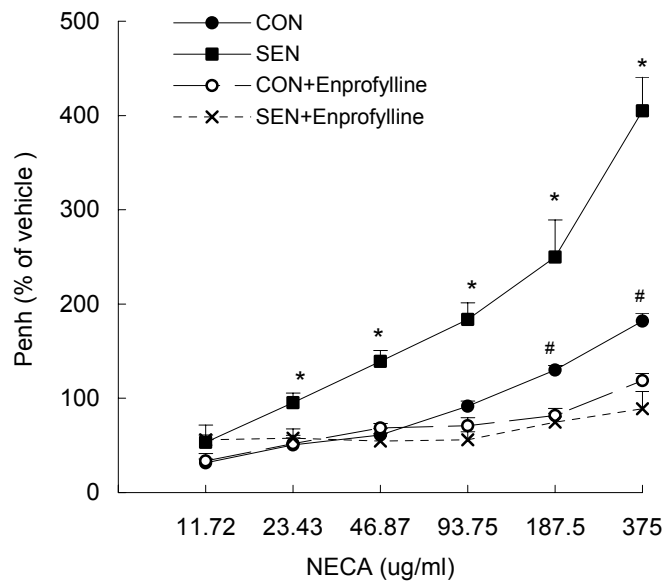


Figure 5. Dose-response to NECA without and with pretreatment with enprofylline ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+Enprofylline: control mice pretreated with enrpfyline; SEN+Enprofylline: sensitized mice pretreated with enprofylline. n=8-10 for each group. Values are means  $\pm$  SE. \*  $p < 0.05$ , compared with CON group, #  $p < 0.05$ , compared with CON+Enprofylline group.

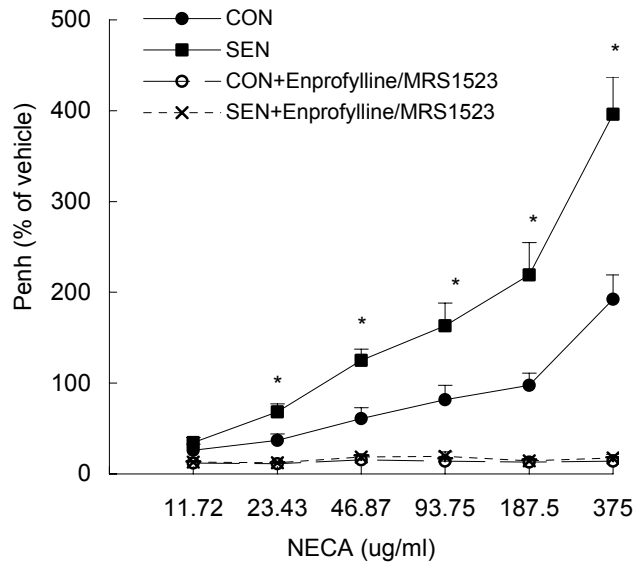


Figure 6. Dose-response to NECA without and with the pretreatment with Enprofylline ( $10^{-4}$  M) plus MRS1523 ( $10^{-4}$  M). CON: control group; SEN: sensitized group; CON+Enprofylline/MRS1523: control mice pretreated with enprofylline plus MRS1523; SEN+Enprofylline/ MRS1523: sensitized mice pretreated with enprofylline plus MRS1523. n=8-10 for each group. Values are means  $\pm$  SE. \*  $p < 0.05$ , compared with CON group.

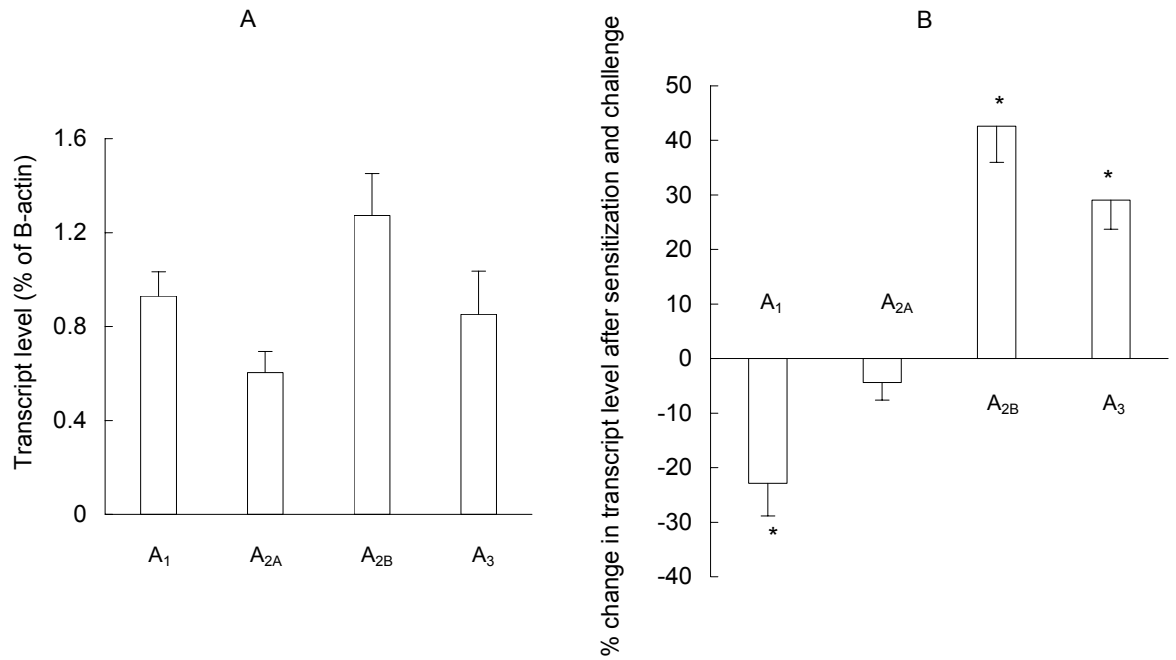


Figure 7. A: Transcription level for adenosine receptors in control mouse lung. B: Percentage changes in transcription level for adenosine receptors in lungs after allergen sensitization and challenge of mice. n=12-19 for each subtype of adenosine receptors. Values are mean +/-SE. \* p<0.05, compared with control mice.