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Involvement of A₁ adenosine receptors in altered vascular responses and inflammation in an allergic mouse model of asthma

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Ponnoth DS, Nadeem A, Tilley S, Mustafa SJ. Involvement of A₁ adenosine receptors in altered vascular responses and inflammation in an allergic mouse model of asthma. *Am J Physiol Heart Circ Physiol* 299: H81–H87, 2010. First published April 16, 2010; doi:10.1152/ajpheart.01090.2009.—Poor lung function and respiratory disorders like asthma have a positive correlation with the development of adverse cardiovascular events. Increased adenosine levels are associated with lung inflammation that could lead to altered vascular responses and systemic inflammation. We hypothesized that asthmatic lung inflammation has systemic effects through A₁ adenosine receptors (A₁AR) and investigated the effects of aerosolized adenosine on vascular reactivity and inflammation, using A₁AR knockout (A₁KO) and corresponding wild-type (A₁WT) mice that were divided into three experimental groups each: control (CON), allergen sensitized and challenged (SEN), and SEN + aerosolized adenosine (SEN + AD). Animals were sensitized with ragweed (200 µg ip; days 1 and 6), followed by 1% ragweed aerosol challenges (days 11 to 13). On day 14, the SEN + AD groups received one adenosine aerosol challenge (6 mg/ml) for 2 min, and aortae were collected on day 15. 5'-N-ethylcarboxamidoadenosine (NECA; non-selective adenosine analog) induced concentration-dependent aortic relaxation in the A₁WT CON group, which was impaired in the A₁WT SEN and SEN + AD groups. All groups of A₁KO mice showed similar (no significant difference) concentration-dependent relaxation to NECA. The A₁WT SEN and SEN + AD groups had a significantly higher contraction to selective A₁ agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA) compared with the CON group. Western blot data showed that aortic A₁AR expression was significantly increased in WT SEN and SEN + AD mice compared with CON mice. Gene expression of ICAM-1 and IL-5 was significantly increased in allergic A₁WT aorta and were undetected in the A₁KO groups. A₁WT allergic mice had significantly higher airway hyperresponsiveness (enhanced pause) to NECA, with adenosine aerosol further enhancing it. In conclusion, allergic A₁WT mice showed altered vascular reactivity, increased airway hyperresponsiveness, and systemic inflammation. These data suggest that A₁AR is proinflammatory systemically in this model of allergic asthma.

vascular reactivity; airway responsiveness; adenosine

ASTHMA IS A CHRONIC LUNG DISEASE characterized by airway hyperreactivity, inflammation, and poor ventilation. Evidence has increasingly implicated adenosine in the pathophysiology of asthma (53). Increases in adenosine levels correspond to increased airway inflammation and tissue damage (14). The sources of adenosine include platelets, neurons, parenchymal lung cells and endothelial cells, and also inflammatory cells, i.e., mast cells, neutrophils, and eosinophils (32, 35, 48). A

hallmark study done in the early 1980s showed inhaled adenosine to be a potent bronchoconstrictor in patients with asthma as opposed to normal subjects who showed no response (10). Genetically modified mice lacking adenosine deaminase (ADA), the enzyme that breaks down adenosine, had severe pulmonary inflammation, airway hyperreactivity, and airway remodeling (5, 9), indicating a strong correlation between chronic elevation of adenosine levels and increased lung inflammation.

Impaired lung function is a risk factor for cardiovascular disease (57). Many epidemiological studies have reported that people suffering from chronic respiratory diseases, including asthma, have a higher incidence of cardiovascular diseases (13, 27, 30, 50, 58). There is an association between atherosclerosis and stroke with reactive airway diseases. Adult-onset asthma is associated with increased carotid atherosclerosis in women (46), and subjects who had bronchial hyperresponsiveness to methacholine (MCh) demonstrated increased carotid intima-media thickness (60). Systemic inflammation in these patients, believed to be a consequence of airway inflammation (28, 39), may be one of the reasons for altered cardiovascular parameters (21). Studies done in animal models of allergic asthma have also reported enhanced systemic inflammation, myocardial ischemia-reperfusion injury, and neutrophil recruitment to the myocardium (23, 24). A recent study from our laboratory (47) has also shown that allergic mice have poor vascular responses and systemic inflammation, with adenosine aerosol exacerbating these effects.

The four adenosine receptor subtypes, viz., the A₁, A_{2A}, A_{2B}, and A₃, have different modulatory roles in asthma and on the cardiovascular system (4, 37, 52). Many reports suggest that the A₁ adenosine receptor (A₁AR) is involved in the direct bronchoconstrictor effects of adenosine. A₁AR expression was found to be elevated in a rabbit model of asthma, and the use of an A₁ antisense nucleotide to inhibit this upregulation resulted in blunted bronchoconstriction to adenosine (1, 43). The use of a selective A₁ antagonist produced a significant reduction of airway hyperresponsiveness in allergic rabbits by blocking these receptors (44). A very recent study reported that the expression of A₁AR is significantly elevated in airway smooth muscle in asthmatic patients (7). Other than the effects on airway smooth muscle, the A₁AR has been implicated in increased mucin production in human tracheal cells (36) and in mediating monocyte phagocytosis (49). All these studies suggest a strong role for the A₁AR in asthma, both in airway obstruction and inflammation.

A₁ARs also have systemic effects and are involved in blood pressure regulation (8) and vasoconstriction (22, 51, 54, 55). However, the role of A₁ARs in the control of vascular tone and

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systemic inflammation in an allergic model of asthma has not been characterized. In addition, the vascular effects of A₁ARs in response to aerosolized adenosine challenge as an added insult in a murine model of asthma have not been studied previously. Therefore, this study was undertaken to investigate the effects of allergen challenge and inhaled adenosine on vascular reactivity and systemic inflammation using our murine model of asthma in A₁AR wild-type (A₁WT) and knockout (A₁KO) mice. We also determined the role of the A₁AR in airway responsiveness by whole body plethysmography. Our data suggest that allergic A₁WT mice have altered peripheral vascular reactivity, increased bronchoconstriction, increased aortic expressions of inflammatory cytokine IL-5, and vascular adhesion molecule ICAM-1, with inhaled adenosine further exacerbating these effects. These effects were not observed in A₁KO mice.

MATERIALS AND METHODS

Animals. A₁KO mice on an inbred BALB/c background were generated by backcrossing A₁ heterozygotes (originally 129/OlaHsd background provided by Dr. Bertil Fredholm) with 12 generations to the BALB/c strain. BALB/c control mice (A₁WT) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). A₁KO and A₁WT males 8–10 wk of age were used in all experiments. 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 West Virginia University.

Animal sensitization. Allergen (ragweed) sensitization and challenge was performed according to the protocol described earlier from this laboratory (16–18, 38, 45). This model of allergic asthma has been shown to develop airway inflammation and airway hyperreactivity to MCh. The study comprised six groups of animals: 1) A₁WT control (A₁WT CON); 2) A₁WT allergen sensitized and challenged (A₁WT SEN); 3) A₁WT allergen sensitized and challenged and further challenged with 6 mg/ml of adenosine aerosol for 2 min on day 14 (A₁WT SEN + AD); 4) A₁KO control (A₁KO CON); 5) A₁KO allergen sensitized and challenged (A₁KO SEN); and 6) A₁KO allergen sensitized and challenged and further challenged with 6 mg/ml of adenosine aerosol for 2 min on day 14 (A₁KO SEN + AD). Experiments were also carried with A₁WT CON mice subject to adenosine challenge (A₁WT CON + AD), and these had no differences from A₁WT CON for vascular reactivity in this study (data not shown) as well as in a previous study from our laboratory (47); hence, this group was not included for further experiments. Mice were sensitized on days 1 and 6 with intraperitoneal injections of ragweed allergen (200 µg/dose; Greer, Lenoir, NC) with 200 µl Imject alum (Pierce; Rockford, IL). Nonsensitized control animals received only the Imject alum with the same volumes. Ten days after sensitization, the mice were placed in a Plexiglas chamber and challenged with 1% 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 3 consecutive days. The aerosolization of allergen was performed at a flow rate of 2 ml/min, and the aerosol particles had a median aerodynamic diameter of <4 µm (DeVilbiss).

Acute elevations in lung adenosine levels were produced experimentally by adenosine inhalation (6 mg/ml for 2 min on day 14) to the SEN + AD group to further enhance the allergen-induced effects. This dose was chosen based on our previous studies in this model (16, 17). Adenosine inhalation in this model has been shown to enhance allergen-induced airway inflammation and airway hyperreactivity to adenosine [or its analog 5'-N-ethylcarboxamidoadenosine (NECA)] (16–18). ADA-deficient mice having sustained and chronic elevations in lung adenosine levels have also been shown to have similar features. The CON and SEN groups received only saline on day 14.

Twenty-four hours after the last challenge, animals were euthanized for the collection of aorta for further experiments.

Preparation of isolated mouse aorta and isometric force measurement. Mice were euthanized by anesthesia with pentobarbital sodium (65 mg/kg ip), followed by thoracotomy and removal of aorta that was then cut transversely into 3- to 4-mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10-ml organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4) containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose, and 2.5 CaCl₂ was maintained at 37°C with continuous bubbling of 95% O₂ and 5% CO₂. For the measurement of isometric force response, the aortic rings were equilibrated for 90 min with a resting force of 1 g and a change of the bathing solution at 15-min intervals. The resting force of 1 g has been used earlier in our laboratory (2, 55). At the end of equilibration period, tissues were contracted with 50 mM KCl twice to check the contractility of individual aortic rings that were washed out with Krebs-Henseleit buffer. The aortic rings were then precontracted with phenylephrine (PE, 10⁻⁷ M) to obtain a steady contraction, and changes in tension were continuously monitored with a fixed range precision force transducer (TSD, 125 C, Biopac system) connected to the differential amplifier (DA 100B, Biopac system). The data were recorded using MP100, Biopac digital acquisition system, and analyzed using Acknowledge 3.5.7 software (Biopac system).

Contraction/relaxation experiments. After equilibration, the responsiveness and stability of individual rings were checked by successive administration of submaximally effective concentration of PE (10⁻⁷ M). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10⁻⁷ M) to produce the relaxation of PE-precontracted rings. The aortic rings were then washed several times with Krebs-Henseleit solution and allowed to equilibrate for 30 min before the experimental protocol began.

Experimental protocol. The concentration-response curves for NECA (10⁻¹¹–10⁻⁵ M) and 2-chloro-N⁶-cyclopentyladenosine (CCPA; 10⁻¹¹–10⁻⁵ M), respectively, were run parallel in aortic rings from all the groups. In all cases, drug was added to yield the next higher concentration only when the response to the earlier dose reached a steady state. Contraction/relaxation responses were expressed as a percent increase/decrease in the contraction with respect to PE (alone) in response to each concentration of agonist used.

Western blot analysis for aortic A₁AR expression. Aorta from the CON, SEN, and SEN + AD A₁WT mice were isolated, and each sample was treated with 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.25% sodium deoxycholate, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM Na₃VO₄, and 1 mM NaF and homogenized on wet ice. The samples were transferred to dry ice for 5 min and then thawed on wet ice. After being thawed, the samples were vortexed and centrifuged for 5 min at 12,000 rpm at 4°C. The supernatants were sonicated and stored at -80°C. Protein was measured using Bio-Rad assay based on the Bradford dye-binding procedure with bovine serum albumin as a standard. The protein mixture was divided into aliquots and stored at -80°C. At the time of analysis, the samples were thawed and ~40 µg of total protein per lane were loaded on a slab gel. Proteins were separated by SDS-PAGE using 10% acrylamide gels (1 mm thick). After electrophoresis, the proteins on the gel were transferred to nitrocellulose membrane (Hybond-ECL) by electroelution. Protein transfer was confirmed by employing prestained molecular weight markers (Bio-Rad; Hercules, CA). Following the blocking with nonfat dry milk, the nitrocellulose membranes were incubated with polyclonal antibody cross-reacting with primary antibody for A₁AR (Sigma; St. Louis, MO); β-actin antibody (Santa Cruz Biotechnology; Santa Cruz, CA) was used as an internal control to normalize the target protein expression in each lane. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were developed using enhanced chemiluminescence (Amersham BioSciences) and exposed to X-ray film for

the appropriate time. The data were presented as the ratio of A₁AR expression to β -actin.

Real-time PCR for expression of ICAM-1, VCAM-1, and IL-5 genes. The aortic tissues from all experimental groups were processed for total RNA isolation using the TRIzol reagent from Life Technologies/Invitrogen followed by DNase treatment to eliminate potential genomic DNA contamination as described recently by us (2, 56). This was followed by the conversion of 0.5 μ g of total RNA into cDNA using High-Capacity cDNA archive kit (Applied Biosystems; Foster City, CA) in a total volume of 100 μ l. Real-time PCR was then performed using an ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems; Branchburg, NJ) according to the instructions of the manufacturer. The reaction volume (25 μ l) consisted of 12.5 μ l of 2 $\times$ Taqman Universal Mastermix, 1 μ l of cDNA, and 1.25 μ l of 20 $\times$ FAM-labeled Taqman gene expression assay master mix solution. For the real-time PCR of the concerned genes (ICAM-1, VCAM-1, and IL-5), the Taqman inventoried assays-on-demand gene expression products were purchased from Applied Biosystems (Foster City, CA). 18S rRNA (ribosomal RNA) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative CT method as described earlier (31).

Airway responsiveness to MCh and NECA. The airway responsiveness was assessed by whole body plethysmography using the chambers obtained from Buxco (Max II; Buxco Systems; Troy, NY) as described previously by our laboratory (40). This system enables the measurement of airway obstruction using a dimensionless parameter called enhanced pause (P_{enh}). The higher the P_{enh} values, the greater is the airway obstruction. P_{enh} has been shown to correlate with airway resistance and dynamic compliance (29). Unrestrained mice were placed in individual Plexiglas chambers 24 h after the last allergen challenge and subjected to increasing concentrations of MCh (1.5–48.0 mg/ml) to obtain a dose response. NECA dose response was performed 24 h after the MCh experiment. After the baseline and vehicle P_{enh} values were obtained, the mice were exposed to increasing concentrations of NECA (23.44–750 μ g/ml) via the Buxco aerosol delivery system (version 1.5; Buxco; Sharon, CT). Each concentration of NECA was aerosolized for 2 min, and the P_{enh} readings were recorded for 5 min to establish a dose-response relationship. The next dose was not administered until the mice had returned to baseline P_{enh} levels. Airway responsiveness was normalized to the vehicle control and expressed as a percent increase in P_{enh} .

Drugs. Acetylcholine, adenosine, and PE were dissolved in distilled water. NECA and CCPA were dissolved in 100% DMSO as a 10 mM stock solution for vascular reactivity experiments. The final concentration of DMSO in organ bath (10 ml) had no effect by itself on the aortic rings (2). For P_{enh} responses, MCh was dissolved in 0.9% saline and NECA was dissolved in 100% ethanol to create a stock solution from which subsequent dilutions were made with 0.9% saline. Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals.

Statistical analysis. The data were expressed as means $\pm$ SE. Comparisons among different groups were analyzed by ANOVA, followed by Tukey's multiple comparison tests. Comparison between two groups was assessed by unpaired *t*-test. A *P* value of <0.05 was considered as the level of significance. All statistical analyses were performed using GraphPad Prism statistical package.

RESULTS

Vascular reactivity in CON, allergic, and allergic + AD groups. The aortic vascular response to NECA was studied in all experimental groups. Organ bath data showed a concentration-dependent relaxation response to NECA in the A₁WT CON group ($48.64 \pm 1.93\%$ at 10^{-5} M; $n = 10$), whereas relaxation was significantly lower in A₁WT SEN aorta (max-

imum relaxation of $22.8 \pm 4.9\%$ at 10^{-5} M; $n = 10$). NECA failed to elicit any relaxation response in the A₁WT SEN + AD group except at the highest concentration ($17.4 \pm 5.53\%$ at 10^{-5} M; $n = 10$, Fig. 1A). In contrast, all three groups of KO mice showed a concentration-dependent relaxation (maximum, $\sim 50\%$ at 10^{-5} M), and there was no difference in the response seen among the groups (Fig. 1B). CCPA, a selective A₁ agonist, produced significantly higher contraction in the A₁WT SEN and SEN + AD groups (44.44 ± 4.12 and $46.2 \pm 5.4\%$, respectively, vs. $10.62 \pm 3.39\%$ in A₁WT CON at 10^{-6} M, Fig. 2; $n = 6$). These data show that allergen sensitization and challenge leads to altered vascular reactivity in allergic A₁WT mice, which suggests that the A₁AR is responsible for the contractile responses in aorta from allergic mice and those challenged with adenosine aerosol.

Western blot analysis for aortic A₁AR expression. Protein expression of A₁AR (~ 37 kDa) was determined by Western blot analysis in all three groups (CON, SEN, and SEN + AD) of A₁WT mice. Densitometric analysis revealed that the SEN ($P < 0.05$; $n = 3$) and SEN + AD ($n = 3$; $P < 0.05$) mice had a significantly higher expression of A₁AR compared with CON mice (Fig. 3). These data suggest that the upregulation of the

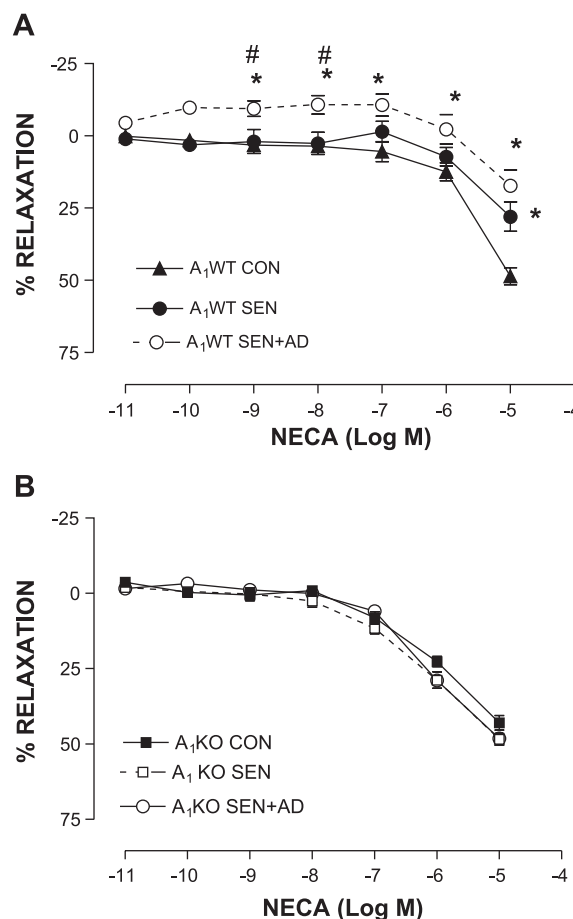


Fig. 1. Concentration-response curves for 5'-N-ethylcarboxamidoadenosine (NECA)-mediated aortic relaxation/contraction in A₁ adenosine receptor (A₁AR) wild-type (A₁WT; A) and A₁AR knockout (A₁KO; B) with control (CON), allergic (SEN), and adenosine aerosolized allergic (SEN + AD) groups. Values are expressed as means $\pm$ SE; $n = 10$ mice. On y-axis, positive and negative values indicate relaxation and contraction, respectively. **P* < 0.05 compared with A₁WT CON; #*P* < 0.05 compared with A₁WT SEN.

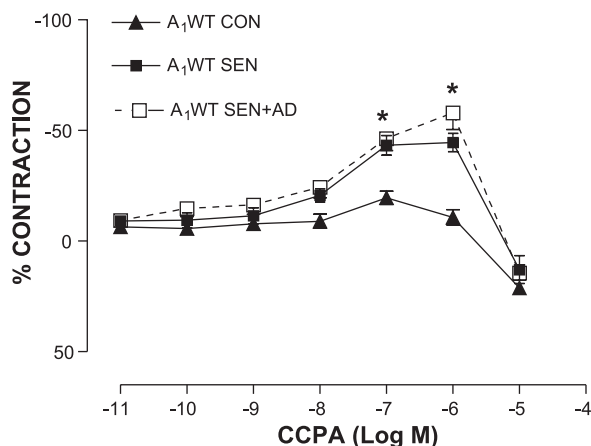


Fig. 2. Concentration-response curves for 2-chloro- N^6 -cyclopentyladenosine (CCPA)-mediated aortic contraction in A_1 WT CON, SEN, and SEN + AD groups. Values are expressed as means $\pm$ SE; $n = 6$ mice. On y-axis, positive and negative values indicate relaxation and contraction, respectively. $*P < 0.05$ compared with A_1 WT CON.

A_1 AR may be responsible for the altered vascular responses observed in allergic mice and those challenged with adenosine aerosol.

Gene expression of vascular adhesion molecules ICAM-1 and VCAM-1, and inflammatory cytokine IL-5 in aorta. Allergen challenge significantly increased levels of vascular adhesion molecule ICAM-1 in A_1 WT SEN compared with A_1 WT CON mice while being undetected in all A_1 KO groups (Fig. 3). Allergen-sensitized and -challenged A_1 WT mice exposed to adenosine aerosol (SEN + AD) had significantly higher levels of ICAM-1 ($P < 0.05$, Fig. 4A) compared with the A_1 WT SEN and A_1 WT CON groups. There was no induction of VCAM-1 gene expression in the A_1 WT and A_1 KO groups in allergic mice and those exposed to adenosine aerosol (data not shown). Inflammatory cytokine IL-5 was significantly higher in the A_1 WT SEN and SEN + AD groups compared with A_1 WT controls (Fig. 4B), whereas IL-5 was not induced in any A_1 KO

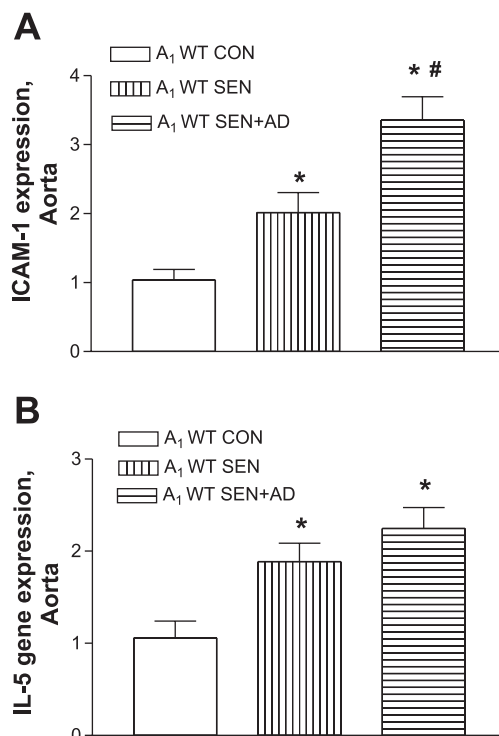


Fig. 4. Expression of ICAM-1 (A) and IL-5 (B) by real-time PCR in A_1 WT mouse aorta from CON, SEN, and SEN + AD groups. For gene expression by comparative C_T method using real-time PCR, first column was made as the calibrator against which all other groups were compared. Values are expressed as means $\pm$ SE; $n = 4$ mice. $*P < 0.05$ compared with A_1 WT CON; $\#P < 0.05$ compared with A_1 WT SEN.

group (data not shown). These data suggest the A_1 AR may have a proinflammatory role in vascular tissue.

Airway responsiveness to MCh and NECA. The airway responsiveness to MCh (Fig. 5) was assessed, and there was no difference between control A_1 WT and A_1 KO mice. All groups of sensitized and challenged A_1 WT and KO mice showed significantly increased airway response to MCh compared with respective controls, but there was no difference between them.

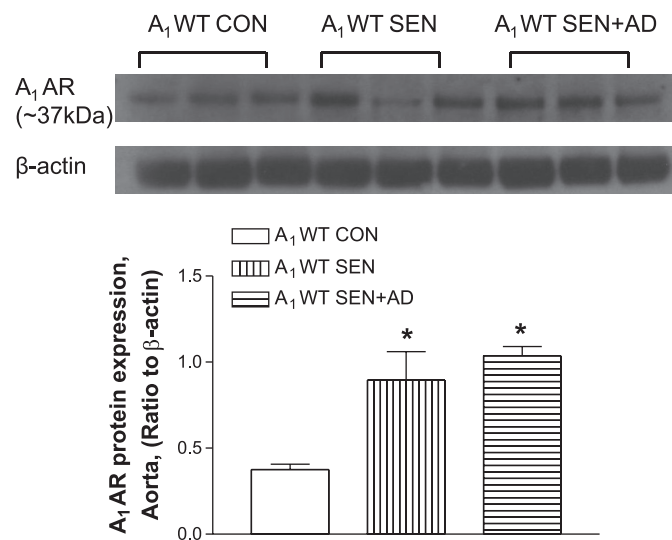


Fig. 3. Protein expression of A_1 AR in mice aorta from A_1 WT CON, A_1 WT SEN, and A_1 WT SEN + AD groups. Values are expressed as means $\pm$ SE; $n = 3$ mice after normalizing A_1 AR protein expression with β -actin in each lane. $*P < 0.05$ compared with A_1 WT CON.

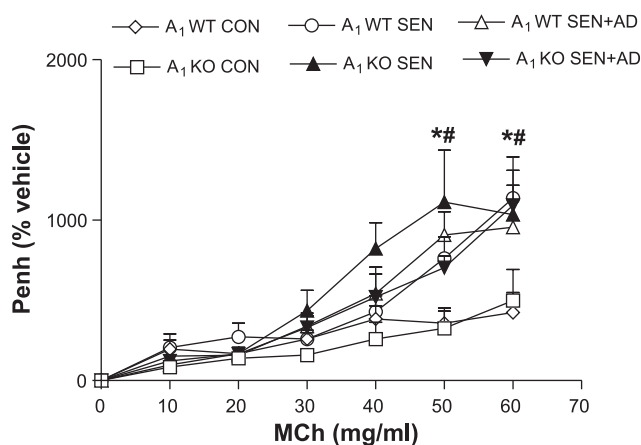


Fig. 5. Airway responsiveness [enhanced pause (P_{enh})] to methacholine (MCh) in A_1 KO and A_1 WT mice. Values are expressed as means $\pm$ SE; $n = 4-8$ mice. On y-axis, P_{enh} is expressed as percentage of the vehicle. $*P < 0.05$, A_1 WT SEN and SEN (S) + AD compared with A_1 WT CON; $\#P < 0.05$, A_1 KO SEN and SEN + AD compared with A_1 KO CON.

The response to NECA was next assessed in all groups (Fig. 6). A₁WT SEN mice had significantly higher airway responsiveness at the maximum dose of NECA (750 μ g/ml) compared with A₁WT CON and all A₁KO groups. The A₁WT SEN + AD group had the highest P_{enh} values compared with the A₁WT SEN, A₁WT CON, and all A₁KO groups. In contrast, the A₁KO SEN, A₁KO SEN + AD, and A₁KO CON groups had similar airway responsiveness to NECA with no differences among them. These data indicate that adenosine-mediated hyperresponsiveness is mediated through A₁AR.

DISCUSSION

The principal finding of the present study is that allergic A₁WT mice had vascular tissue inflammation, increased A₁AR expression, increased airway hyperresponsiveness, impaired vasorelaxation to NECA, a nonselective adenosine analog, and a greater contraction to A₁ selective agonist CCPA. These outcomes were further aggravated after acute adenosine aerosol challenge in these mice. Allergen- and adenosine-induced effects seen in A₁WT SEN and A₁WT SEN + AD mice were absent in the A₁KO groups, indicating that A₁AR is responsible for altered vascular reactivity, increased airway hyperresponsiveness, and increased systemic inflammation.

Adenosine is chronically elevated in asthmatic lungs (14, 33, 34). Evidence indicates that adenosine may be involved in the release of inflammatory mediators in the lung and systemic circulation by acting on its receptors present on different cells including mast cells, eosinophils, neutrophils, and other inflammatory cells (15, 16, 41, 53) and can thus contribute to pulmonary and systemic inflammation. Adenosine is a known bronchoprovocant (11), and experimentally induced temporary elevations in lung adenosine levels through the inhalation of adenosine (resulting from the breakdown of adenosine 5'-monophosphate) cause an increase in the infiltration of eosinophils in patients with asthma (59). A recent study from our laboratory has also shown an increased release of inflammatory cell markers in the lung and plasma after inhalation of adenosine aerosol in allergic mice (17). Adenosine aerosol in humans with asthma also causes an increased release of neutrophil

chemotactic factor in serum (15). These studies suggest that adenosine enhances the release of systemic inflammatory mediators from sensitized inflammatory cells. However, the systemic effects of such an inhalation on vascular tissue inflammation with respect to the involvement of A₁ARs have not been studied previously.

Systemic inflammation has been reported previously in human and animal models of asthma (23, 28). Neutrophils and eosinophils were increased in bronchoalveolar lavage and blood in a murine model of asthma (24). Systemic inflammatory markers have also been shown to be elevated in patients with asthma and in allergic mouse model of asthma after adenosine aerosol challenge (15, 16). Our earlier study (47) has also shown that allergic mice had significantly elevated systemic levels of proinflammatory cytokines, poor endothelial function, and lower aortic relaxation, with acute adenosine aerosol further causing exacerbation of systemic inflammation and producing an impairment of aortic relaxation. The use of A₁ antagonist DPCPX increased aortic relaxation in both sets of experimental groups. These data suggested a possible involvement of A₁ARs in alteration of vascular reactivity and inflammation. The next logical step was to determine whether inflammation was present in aortic tissue of allergic mice and whether A₁AR had any role in this.

We found that the gene expression of ICAM-1 was significantly higher in both groups of allergic A₁WT mice (SEN and SEN + AD) compared with controls. There was no difference in ICAM-1 expression in A₁KO groups (data not shown). Several lines of evidence indicate that a crucial development observed in atherosclerosis is the increased expression of vascular adhesion molecules in the endothelium (6, 20). In the present study, we have observed an increased expression of ICAM-1 correlating well with poor endothelial relaxation reported in our earlier study (42) in allergic A₁WT mice. For the first time, a link has been found among the presence of A₁AR, increased expression of ICAM-1, and endothelial dysfunction in aorta from allergic mice. While both ICAM-1 and VCAM-1 are proinflammatory and controlled by the NF- κ B pathway, evidence suggests that their expression may be differentially regulated (26, 42). Higher levels of ICAM-1 were also observed in obese children with asthma, compared with VCAM-1 levels (25). This could explain why differences in VCAM-1 were not seen in our study.

Gene expression of inflammatory cytokine IL-5 was significantly higher in the A₁WT SEN and A₁WT SEN + AD groups compared with the A₁WT CON group, whereas IL-5 was undetected in all the A₁KO groups. IL-5 is a very important mediator in allergic asthma (19) and is a key player in the induction of eosinophilia (3). Though eosinophilia is considered to be a characteristic hallmark of asthmatic inflammation, there is no previous evidence suggesting a link between asthma and IL-5 in vascular tissue inflammation. In fact, it is likely that several inflammatory cytokines induced by allergen challenge may be responsible for altered vascular responses and may play a part in systemic and vascular inflammation. More studies are needed to determine the signaling pathway responsible for the upregulation of IL-5 through A₁AR in the vasculature of allergic mice.

These findings on ICAM-1 and IL-5 gene expression suggest that the absence of the A₁AR appears to limit adenosine-mediated inflammation in the vasculature. Further studies are

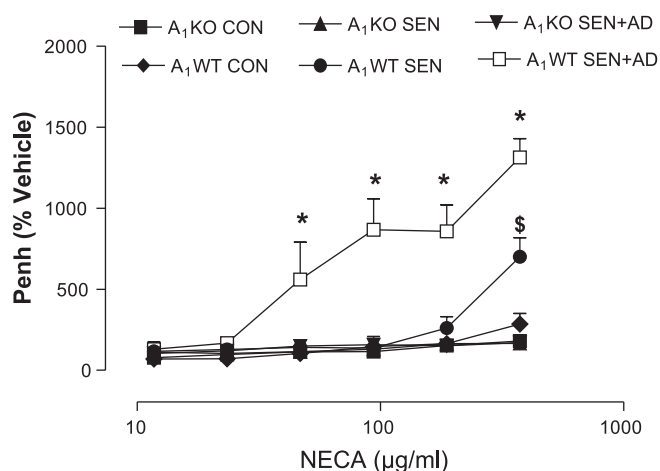


Fig. 6. Airway responsiveness (P_{enh}) to adenosine analog NECA in A₁KO and A₁WT mice. Values are expressed as means $\pm$ SE; n = 4–8 mice. On y-axis, P_{enh} is expressed as percentage of the vehicle. * P < 0.05 compared with all other groups; \$ P < 0.05 compared with A₁WT CON and all A₁KO groups.

required to delineate the exact mechanism for the role of the A₁AR in the development of these adverse vascular events observed in our model of asthma.

Asthma has been associated with an increased incidence of negative cardiovascular outcomes (13, 27, 30, 58). While asthmatic inflammation is believed to be responsible for the development of these cardiovascular effects, there is little information linking the two, especially in relation to the vascular effects of A₁AR. In the present study, NECA, a nonselective adenosine analog, produced a concentration-dependent vasorelaxation in A₁WT CON mice, which was impaired in allergic A₁WT mice. Vascular reactivity to NECA was further altered in aorta of allergic A₁WT mice that had received aerosolized adenosine. On the other hand, NECA produced a concentration-dependent vasorelaxation response in allergic and nonallergic A₁KO groups. We also used the selective A₁ agonist CCPA to further confirm the role of A₁AR in altered vascular reactivity. CCPA elicited a significantly higher contraction in allergic A₁WT mice, thus underscoring the involvement of A₁AR. Western blot analysis showed that the expression of A₁AR was significantly higher in the WT SEN and SEN + AD groups compared with CON. These findings strongly suggest that the A₁AR indeed is responsible for the altered vascular responses in allergic and adenosine aerosolized allergic WT mice. Whereas adenosine aerosol exacerbated the NECA response in A₁WT SEN + AD compared with A₁WT SEN mice, no difference was observed in the CCPA response. Previously, we found that the aortic expression of A_{2A} and A_{2B} receptors, which are involved in vasorelaxation, was lower in the SEN + AD group (47), and this could possibly explain the difference in the NECA response in the present study.

Poor lung function is associated with an increased risk of cardiovascular disease (51). Whole body plethysmography has been used previously to determine the extent of airway hyperresponsiveness and as a measure of airway obstruction (12, 17, 18, 45). We used this method to determine the differences between A₁WT and A₁KO responses to MCh and NECA. MCh produced higher airway responsiveness in both A₁WT- and A₁KO-sensitized and -challenged animals compared with the respective controls. No difference was observed between different groups of allergic A₁WT and A₁KO mice. However, airway responses to the nonselective adenosine analog NECA were different between A₁KO and A₁WT mice, suggesting the involvement of A₁AR. There was no difference in NECA-mediated airway responsiveness in all three groups of A₁KO mice; on the other hand, the A₁WT SEN group had significantly higher bronchoconstriction (P_{enh}) compared with the A₁WT CON group. The highest bronchoconstrictor response was observed in the A₁WT SEN + AD group, which was significantly different from all other study groups. A₁AR has been implicated in the adenosine-mediated bronchoconstrictor response earlier (1, 43), and selective A₁AR antagonist has been shown to produce a significant reduction of airway hyperresponsiveness in allergic rabbits by blocking these receptors (44). Our data support a role for A₁AR-mediated airway hyperresponsiveness and also show that adenosine aerosol challenge exacerbates this response in allergic mice.

In conclusion, our findings provide evidence for the first time that the A₁AR is involved in altered vascular tissue parameters (aortic inflammation, impaired vasorelaxation, and

increased A₁AR expression) and airway hyperresponsiveness as a result of allergen exposure and adenosine aerosol challenge in sensitized mice. Understanding the exact mechanism of A₁AR-mediated signaling would provide an important tool in the prevention of systemic inflammation and impaired vascular responses observed in people with asthma. Ultimately, this could be beneficial in lowering cardiovascular events in patients with asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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