Articles in PresS. Am J Physiol Lung Cell Mol Physiol (February 9, 2007). doi:10.1152/ajplung.00416.2006

Enhanced airway reactivity and inflammation in A_{2A} adenosine receptor deficient allergic mice

Ahmed Nadeem[1], Ming Fan[2], Habib R Ansari[1], Catherine Ledent[3] and S. Jamal Mustafa[1]*

[1]Department of Physiology and Pharmacology, School of Medicine, West Virginia University, Morgantown, WV; [2]Department of Pharmacology, East Carolina University, Greenville, NC; [3] Institut de Recherche Interdisciplinaire en Biologie Humaine et Nucléaire, Université Libre de Bruxelles, Brussels, Belgium

Running Head: A_{2A} adenosine receptor, nitrosative stress and asthma

Correspondence:

*Dr. S. Jamal Mustafa

West Virginia University

Morgantown, WV 26505-9229

Email: smustafa@hsc.wvu.edu

Telephone: 304-293-5830

Fax: 304-293- 7038

ABSTRACT

A_{2A} adenosine receptor (A_{2A}AR) has potent anti-inflammatory properties, which may be important in the regulation of airway reactivity and inflammation. Inflammatory cells that possess A_{2A}AR also produce nitrosative stress which is associated with pathophysiology of asthma, so we hypothesized that A_{2A}AR deficiency may lead to increased airway reactivity and inflammation through nitrosative stress. Thus, the present study was carried out to investigate the role of A_{2A}AR on airway reactivity, inflammation, nuclear factor kappa B (NF-kB) signaling and nitrosative stress in A_{2A} AR knock-out (KO) and wild-type (WT) mice using our murine model of asthma. Animals were sensitized i.p. on days 1 and 6 with 200µg of ragweed, followed by aerosolized challenges with 0.5% ragweed on days 11, 12 and 13, twice a day. On day 14, airway reactivity to methacholine was assessed as enhanced pause (Penh) using whole body plethysmography followed by bronchoalveolar lavage (BAL) and lung collection for various analyses. Allergen challenge caused significant decrease in expression of A_{2A}AR in A_{2A}WT sensitized mice, with A_{2A}AR expression being undetected in A_{2A}KO sensitized group leading to decreased lung cAMP levels in both groups. A_{2A}AR deletion/downregulation led to an increase in Penh to methacholine and influx of total cells, eosinophils, lymphocytes and neutrophils in BAL with highest values in A_{2A}KO sensitized group. A_{2A}KO sensitized group further had increased NF-kB expression and nitrosative stress as compared to WT sensitized group. These data suggest that A_{2A}AR deficiency leads to airway inflammation and AHR, possibly via involvement of nitrosative stress in this model of asthma.

Key words: A_{2A} adenosine receptors, Asthma, Inflammation, Airway reactivity, Nitrosative stress

INTRODUCTION

Asthma is an inflammatory disease of the airways characterized by reversible airflow obstruction, bronchitis, and airway hyperresponsiveness (AHR). The pathogenesis of asthma involves infiltration of various inflammatory cells such as neutrophils, mast cells, lymphocytes and eosinophils, which secrete a number of mediators including reactive oxygen/nitrogen species (3, 7, 31).

Adenosine is a potent signaling nucleoside that plays important roles in the regulation of homeostasis in the lung and has been implicated in asthma (8). The mechanisms of the adenosine-mediated bronchoconstriction and inflammation are through G-protein-coupled receptors on the target cells. Four subtypes of adenosine receptors namely A_1, A_{2A}, A_{2B}, and A_3, have been identified. Each receptor has unique tissue distribution, ligand affinity, and signal transduction pathways (14).

Strong anti-inflammatory properties for A_{2A} adenosine receptors (A_{2A} AR) have recently been described in various systems (26, 36). The A_{2A} AR is expressed on inflammatory cells, including neutrophils, mast cells, lymphocytes, macrophages, and monocytes (27, 44). Activation of A_{2A} AR causes reduction in chemotaxis, phagocytosis, the adherence of neutrophils to endothelial cells, and the secretion of pro-inflammatory cytokines, thus showing anti-inflammatory properties (24). Recent studies have also emphasized its anti-inflammatory effects in models of respiratory disorders and in restoration of bronchial injury (1, 4, 13). However, relatively little is known about the biochemical and molecular mechanisms by which A_{2A} AR causes its pathophysiological actions in asthma. Recently, the activation of A_{2A}ARs has also been shown to exert inhibitory effects on NF-kB signaling *in vitro* (5, 30, 43).

Since inflammatory cells possess A_{2A}ARs (24) and at the same time also have the capacity to generate nitrosative stress by producing several reactive oxygen/nitrogen species (2, 31, 32). In such a context, the production of nitric oxide (NO) is important as it can lead to the generation of highly reactive intermediates such as peroxynitrite capable of oxidizing lipids and nitrating proteins (3-nitrotyrosine) (2, 40). It is, therefore, possible that the protective effect of A_{2A}ARs is mediated through attenuation of nitrosative stress possibly via suppression of iNOS expression and 3-NT formation. A_{2A}AR has been shown to inhibit the generation of reactive oxygen species in leukocytes (45) and expression of iNOS *in vitro* (43). On the other hand, increased nitrosative stress due to deficiency of A_{2A}AR can lead to depletion of endogenous non-enzymatic antioxidants such as vitamin E, ascorbic acid, and protein thiols present in the lung (15, 19), which collectively form total antioxidant capacity (TAC) and are negatively correlated with airway obstruction in asthmatics (31, 32).

Thus, the present study was conducted to assess the role of A_{2A}ARs on airway reactivity, inflammation and nitrosative stress using A_{2A}AR knock-out mice.

MATERIALS AND METHODS

Mice Sensitization and Challenge

A_{2A}KO and WT mice were obtained from the Institute of Experimental Medicine (C. Ledent, Universite Libre de Bruxelles, Brussels, Belgium). To homogenize the genetic background of the mice, the first generation heterozygotes were bred for 14 generations on a CD1 (Charles River, France) outbred background, with selection for the mutant A_{2A} gene at each generation by PCR. Fourteenth-generation heterozygotes were bred together to generate the KO A_{2A} and WT.

Male A_{2A} KO and their WT littermate controls, 11 to 14 weeks of age, free of specific pathogens, were used in the 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.

Sensitization was performed according to the protocol described earlier from this laboratory (11, 12). Mice were sensitized on days 1 and 6 with i.p. injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 μg per dose with 200 μl Imject® Alum (Pierce Laboratories, Rockford, IL). Non-sensitized control animals only received the Imject® alum with the same volumes. 10 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 minutes 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 had a median aerodynamic diameter of less 4 μm (De Vilbiss).

Mice were divided into following groups: wild-type control group (A_{2A}WT Control): mice received only vehicles for sensitization and challenge; wild-type sensitized group (A_{2A}WT Sensitized): mice were sensitized and challenged with ragweed using the same protocol described above; knock-out control group (A_{2A}KO Control): mice received only vehicle for sensitization and challenge; knock-out sensitized group (A_{2A}KO Sensitized): mice were sensitized and challenged with ragweed using the same protocol described above.

Measurement of Airway Reactivity in vivo

Twenty-four hours after final allergen aerosol exposure, airway reactivity to methalcholine in conscious, unrestrained mice were assessed by a whole-body noninvasive plethysmograph (Buxco Electronics, Troy, NY) as described earlier from this laboratory (11, 12). This system estimates total pulmonary airflow in mice using a dimensionless parameter known as enhanced pause (Penh). Pressure differences were used to extrapolate Penh values, which are a function of the sum of the airflows in the upper and lower respiratory tracts during a respiratory cycle. This parameter has been shown by us previously to correlate with airway resistance measured by invasive techniques (21). Baseline Penh was determined by exposing mice to nebulized saline. The mice were then exposed to increasing concentrations of aerosolized methacholine dissolved in saline for 1.5 min and then recording and averaging Penh values for 5 min following each nebulization.

Bronchoalveolar Lavage (BAL)

Mice were sacrificed by i. p. injection (0.1 ml pentobarbitone sodium 200 mg/ml). The trachea was cannulated to perform BAL; 0.6 ml phosphate-buffered saline (PBS)

was introduced into the lungs via the tracheal cannula and carefully withdrawn. This was repeated three additional times to collect remaining cells. The recovered fluid (75 to 80% of the injected volume) was centrifuged at 800 *g* for 10 min at 4° C and the resulting supernatant was stored at −80° C for total antioxidant status and lipid peroxidation assays. After resuspension in PBS, the total cells were counted manually in a hemocytometer chamber (Fisher). 1~5 × 10^3 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 ± SE per ml for each group.

For immunocytochemistry, BAL slides were fixed in acetone-methanol (ratio of 6:4) at room temperature (RT) for 7 min and stored at −20° C after 1 h air-drying. After the BAL procedure, the right lung was homogenized with six volumes of ice-cold tissue lysis buffer consisting of 0.05 M Tris-buffered saline (TBS) pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mmol/l sodium chloride, mmol/l EDTA, mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mmol/l sodium orthovanadate, and 1 mmol/l sodium fluoride (Sigma). Homogenized samples were centrifuged for 30 min at 12,000 × *g* at 4° C and lung supernatant was stored at -80° C for western blot experiments, cAMP, total nitrates and nitrites (NOx) and protein assays.

Biochemical Assays

Total antioxidant capacity assay

Total antioxidant capacity was measured in cell-free BAL fluids according to

Miller and coworkers (29) using a commercial kit (Cayman Chemical Company, Ann Arbor, MI). The antioxidant capacity of BAL was then compared with Trolox as "Trolox equivalent antioxidant capacity" (TEAC). The TEAC in BAL samples was defined as the concentration (µmol/ml) of Trolox having the equivalent antioxidant capacity to 1 ml BAL fluid. This kit measures mainly non-enzymatic antioxidants in the biological fluids. Results were expressed in µmol/l.

Lipid peroxidation assay

Cell membrane damage was monitored through the measurement of malondialdehyde (MDA), a metabolite resulting from lipid peroxidation, which was detected by the method of Jentzsch et al. (20). Briefly, 0.2 ml of BAL supernatant was incubated with 5 mmol/l butylated hydroxyl toluene, 0.2 mol/l ortho-phosphoric acid and 0.11 M TBA in a total volume of 500 µl at 90°C for 45 min, followed by ice-cooling and extraction of MDA-TBA adducts in n-butanol. Absorption was read at 535 and 572 nm for baseline correction in a multititer plate reader. MDA-TBA adducts were calculated using the difference in absorption at the two wavelengths compared to the standard curve generated by the use of tetraethoxypropane. Results were expressed in µmol/l.

Total nitrites and nitrates (NOx) assay

Nitric oxide production in lung homogenate supernatant was detected as total nitrates and nitrites (NOx) by the method of Grisham et al. (16), which are the end products of nitric oxide metabolism and provide one of the most useful methods to quantify nitric oxide production (16). The method is based on the conversion of all the nitrates present in lung supernatant into nitrites in the presence of aspergillus nitrate reductase coupled with NADPH and FAD. Assay mixture contained the sample, 0.86

mmol/l NADPH, 0.11 mmol/l FAD, 20 mU nitrate reductase in 310 mmol/l potassium phosphate buffer (pH-7.5) in total assay volume of 100 µl. Samples were allowed to incubate at 37°C for 2 h in the dark, followed by addition of 1M zinc sulfate to precipitate the proteins. After centrifugation, 50 µl supernatant from each microtube was transferred into individual wells of 96-well microplate, followed by addition of 100 µl Griess reagent (1:1 mixture of 1 % (w/v) sulfanilamide in 5 % (v/v) ortho-phosphoric acid and 0.1 % (w/v) N-(1-naphthyl) ethylene diamine) for color development. Readings were taken after 10 min at 540 nm on a multititer plate reader. Standard curve was generated using known concentrations of sodium nitrite. Results were expressed as nmol/mg protein.

Immunocytochemistry for 3-nitrotyrosine (3-NT) and inducible nitric oxide synthase (iNOS)

After 5 min washing in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH 7.8), defrosted coverslips with BAL cells were incubated with an anti-3NT rabbit polyclonal IgG (1:100 dilution; Upstate Biotechnology, Lake Placid, NY) and anti-iNOS rabbit monoclonal IgG (1:70 dilution; Transduction Laboratories, Lexington, KY) primary antisera diluted in PBS-Tx-BSA, pH 7.8 in a humid chamber at 4°C overnight. They were then rinsed 3 times with PBS-Tx-BSA, allowing 5 min per rinse, and then covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) (Zymed, San Francisco, CA) for 3-NT and rhodamine-conjugated goat anti-rabbit IgG (Upstate, Lake Placid, NY) for iNOS diluted 1:100 in PBS-Tx-BSA and incubated at 37°C for 45 min. After that, the coverslips were rinsed 3 times in PBS-Tx-BSA and were mounted on glass slides in Fluoromount (Southern Biotechnology, Birmingham, AL).

Following immunocytochemistry, the coverslips were mounted with Fluoromount and observed with an Olympus AX70 fluorescence microscope (Olympus America, Melville, NY) equipped with fluorescein (excitation wavelengths 455–500 nm, emission wavelengths >510 nm) and rhodamine (excitation 540–504 nm, emission >580 nm). Nonspecific background labeling was determined by omission of primary antisera. Moreover, negative control for 3-NT included absence of staining without primary antiserum, absence of staining with preincubation with 500 mmol/l sodium hydrosulfite (dithionite) dissolved in 100 mmol/l sodium borate (Sigma) that reduces 3NT to aminotyrosine.

Western Blot for A_{2A} adenosine receptor (A_{2A} AR) and phosphorylated inhibitory protein kappa B-alpha (phospho-IkBα)

Aliquots of the lung supernatant (40 μg protein/well) were separated on 10% SDS-PAGE. Prestained protein molecular markers (20- to 112-kDa low range) were run in parallel. Proteins were transferred to nitrocellulose membranes and then probed with either anti-phospho-IkBα mouse monoclonal IgG (Cell Signaling Technology, Inc., Danvers, MA) for detection of active form of IkBα or anti-A_{2A} AR polyclonal rabbit IgG (28) developed in our laboratory for detection of A_{2A} AR expression diluted 1:1,000, followed by an incubation with the secondary HRP-conjugated antibodies (anti-mouse and anti-rabbit immunoglobulins from goat for phopho-IkBα and A_{2A} AR respectively; Amersham Pharmacia Biotech, Inc.) for 1 h at room temperature. For detection of bands, the membranes were treated with enhanced chemiluminescence (ECL) reagent (Amersham Biosciences) for 1 min and subsequently exposed to ECL Hyperfilm. Relative band intensities were quantified by densitometry (Alpha Innotech Corp., San

Leandro, CA). Western blot values are expressed as % of control after densitometric analysis.

Real-time PCR for adenosine receptors (AR), inducible nitric oxide synthase (iNOS) and p-65 subunit of nuclear factor kappa B (NF-kB)

Total RNA was isolated from the lung using the TRIzol reagent from Life Technologies/Invitrogen followed by DNase treatment to eliminate potential genomic DNA contamination. This was followed by conversion of 0.5 μg of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer in a total volume of 100 μl. Real-time PCR was performed on an ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, Branchburg, New jersey) as described by us in a recent study (17). Briefly, the reaction volume (25 μl) included 12.5 μl of 2X Taqman Universal Mastermix, 1 μl of cDNA, and 1.25 μl of 20X FAM-labeled Taqman gene expression assay master mix solution. For the real-time PCR for all the four adenosine receptors, iNOS and p-65 subunit of NF-kB genes, the Taqman inventoried assays-on-demand gene expression product with GenBank accession nos. NM_001008533 (A_1 AR), NM_009630 (A_{2A} AR), NM_007413 (A_{2B} AR), NM_009631 (A_3 AR), NM_010927 (iNOS), NM_009045 (p-65) were purchased from Applied Biosystems. 18S rRNA (Ribosomal RNA; GenBank accession no. X03205) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative C_T method (25). The ΔC_T value was determined in each group by subtracting the average 18S C_T value from the corresponding average C_T for each gene. The $\Delta\Delta C_T$ of each gene in different groups was calculated by subtracting ΔC_T of first

group (first column in each graph) by ΔC_T of other groups. The fold difference in gene expression of the target was calculated as the average value from $2^{-\Delta\Delta C_T+s}$ and $2^{-\Delta\Delta C_T-s}$.

Measurement of cAMP and protein concentration

cAMP levels and protein levels in lung homogenate were measured using a competitive immunoassay kit from R&D Systems (Minneapolis, MN) and Bradford assay kit from Bio-Rad (Hercules, CA) respectively.

Materials

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

Statistical Analysis

The data were expressed as Mean±SE. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey's multiple comparison test/ Bonferroni's selected pair test. Comparison between two groups was assessed by unpaired t-test. A '*P*' value of less than 0.05 was considered as the level of significance for all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.

RESULTS

Expression of adenosine receptors (ARs) and cAMP levels in the lung after allergen challenge in A_{2A} WT and KO mice

As shown in Fig.1, expression of all the four ARs was found in the lung with A_{2A} AR and A_{2B} AR having the highest expression and A_3 AR having the lowest expression in the A_{2A} WT control mice. Similar pattern was also found in the A_{2A} KO control mice except that no transcripts were detected for A_{2A} AR gene (Fig. 1). Allergen challenge significantly decreased the expression of A_{2A} ARs by 3.5 fold in A_{2A} WT sensitized mice ($P<0.001$) as compared to A_{2A} WT control mice, while there was undetectable expression of these receptors in the A_{2A} KO mice (Fig. 2A). This decreased gene expression was further confirmed by western blot which showed almost 50% less protein expression for A_{2A} AR in A_{2A} WT sensitized group as compared to A_{2A} WT control group ($P<0.05$; Fig. 2B). Decreased expression of A_{2A} ARs in WT sensitized mice and deletion of A_{2A} ARs in KO sensitized mice was associated with 60 and 74% decrease in cAMP levels as compared to the A_{2A} WT and KO control mice, respectively (Fig. 2C; $P<0.01$). Additionally, A_{2A} KO sensitized mice had a further decrease of 48% in cAMP levels as compared to the A_{2A} WT sensitized mice.

Allergen challenge also significantly decreased the expression of A_1 (Fig. 3A), A_{2B} (Fig. 3B) and A_3 ARs (Fig. 3C) in A_{2A} WT ($P<0.01$) and KO ($P<0.01$) sensitized mice as compared to the respective A_{2A} WT and KO control mice. In these groups, A_{2A}

KO sensitized group had the least expression of $A_{1,}$ A_{2B} and A_3 ARs, which were also significantly different from A_{2A} WT sensitized group (Fig. 3 A, B & C; P <0.05).

Leukocyte recruitment to the airways after allergen challenge in A_{2A} WT and KO mice

Recruitment of inflammatory leukocytes to the lung occurs as a consequence of airway challenge with allergen in sensitized mice. The inflammatory cell profile in the BAL is shown in Fig. 4. Total cell number increased significantly in both A_{2A} WT (80%; P <0.05) and A_{2A} KO (190%; P <0.05) sensitized groups after aerosolized ragweed challenge as compared to their respective A_{2A} WT and KO control mice. A_{2A} KO sensitized mice further had an increase of 70% (P <0.05) in total cells as compared to corresponding WT mice (Figure. 4A). Out of the total cells recovered from BAL, eosinophils were the major cell consisting of 29% and 72% in A_{2A} WT and KO sensitized mice, respectively. Eosinophils (Figure. 4B) and lymphocytes (Figure. 4C) increased significantly in both A_{2A} WT (P <0.05) and KO (P <0.05) sensitized groups as compared to their respective A_{2A} WT and KO control mice. A_{2A} KO sensitized group (P <0.05) further had significant difference in both eosinophils and lymphocytes as compared to A_{2A} WT sensitized group. Increase in the number of neutrophils was found only in A_{2A} KO sensitized mice, which was significantly different from both A_{2A} KO control (P <0.05) and A_{2A} WT sensitized (P <0.05) groups (Figure. 4D). On the other hand, there was a slight decrease in the number of macrophages in A_{2A} KO sensitized mice (P <0.05) as compared to A_{2A} WT sensitized mice (data not shown).

Airway reactivity to methacholine after allergen challenge in A_{2A} WT and KO mice

To determine whether deficiency in A_{2A} AR has a direct effect on the

development of airway responsiveness, airway reactivity to methacholine was measured by whole body plethysmography 24 h following the last allergen or saline challenge in A_{2A} WT and KO mice. Fig. 5 shows that there was a significant difference in response to methacholine even in A_{2A} KO control as compared to A_{2A} WT control mice at doses of 3.125 and 12.5 mg/ml (P <0.05). Both A_{2A} WT and KO sensitized mice showed increased Penh following methacholine challenge at all doses as compared to their respective A_{2A} WT and KO control mice (P <0.05). A significant increase in Penh following methacholine challenge was further observed in A_{2A} KO sensitized mice compared with WT sensitized mice at all doses (Fig. 5; P <0.05).

Expression of phosphorylated inhibitory protein kappa B-alpha (phospho-IkBα) and p-65 subunit of nuclear factor kappa B (NF-kB) after allergen challenge in A_{2A} WT and KO mice

Since cAMP is mostly responsible for anti-inflammatory effects of A_{2A} AR, we expected that decreased cAMP levels caused due to downregulation/deletion of A2A ARs might activate NF-kB. In accordance with this, real-time PCR analysis showed 9.7 and 4.6 higher fold expression of p-65 subunit of NF-kB in A_{2A} WT and KO sensitized mice as compared to the A_{2A} WT and KO control mice, respectively (Fig. 6A; P <0.01). Furthermore, A_{2A} KO sensitized group had almost 2 fold higher expression of p-65 as compared to the A_{2A} WT sensitized group (Fig.6A; P <0.05). The most important step in the regulation of NF-kB activity is its nuclear translocation following the release from its inhibitor IkBα, which is achieved by its phosphorylation and subsequent degradation. The experiment in Fig. 6B shows significantly higher phosphorylation of IkBα in A_{2A} WT and KO sensitized groups (P <0.05) as compared to the respective A_{2A} WT and KO

control groups. A_{2A} KO sensitized mice further had significant difference in expression in comparison to A_{2A} WT sensitized mice (P <0.05).

Expression of inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT) and total nitrates and nitrites (NOx) in the lung and BAL cells after allergen challenge in A_{2A} WT and KO mice

NF-kB is thought to be a major transcriptional factor responsible for the upregulation of iNOS. Therefore, downstream effects of NF-kB activation were studied by measuring the expression of iNOS in BAL cells and in the lung as well as 3-NT formation in BAL cells and nitric oxide production as total nitrates and nitrites (NOx) in the lung. Real-time PCR showed that there was an increased expression of iNOS gene in the lung of A_{2A} WT (P <0.05) and KO (P <0.05) sensitized mice as compared to the A_{2A} WT and KO control mice after allergen challenge (Fig. 7A). A_{2A} KO sensitized group further had an increase of 42 % in iNOS expression as compared to A_{2A} WT sensitized group (Fig. 7A). Increase in iNOS expression was associated with increased levels of lung NOx in both A_{2A} WT and KO sensitized mice (Fig. 7B) with values of 1.718± 0.135 nmol/mg protein (n=5; P <0.01) and 2.77± 0.247 nmol/mg protein (n=5; P <0.01), respectively as compared to the A_{2A} WT (0.707± 0.068 nmol/mg protein, n=5) and KO (1.085± 0.144 nmol/mg protein, n=5) control mice. Moreover, A_{2A} KO sensitized mice further had increased levels of lung NOx as compared to the A_{2A} WT sensitized mice (Fig. 7B; P <0.01).

Immunohistochemical analysis of iNOS protein expression and 3-NT formation in BAL cells harvested 24 h later after last allergen challenge revealed immunostaing with both anti-iNOS (Fig. 8 A & B; top panel) and 3-NT (Fig. 8 C & D; bottom panel)

antibodies in A_{2A} WT and KO sensitized groups. The immunostaing was seen much more in KO sensitized BAL cells for both iNOS and 3-NT in comparison to WT sensitized BAL cells. Most of these cells showed 3-NT immunostaing in BAL cells similar in location to iNOS immunostaining, suggesting that increased iNOS may be responsible for 3-NT formation in these cells. On the other hand, only minimal iNOS or 3NT immunostaining was observed in the BAL cells from both WT and KO control mice (data not shown).

Lipid peroxidation and total antioxidant capacity in BAL fluid after allergen challenge in A_{2A} WT and KO mice

Lipid peroxidation and total antioxidant capacity in BAL fluid as markers of nitrosative stress were assessed in A_{2A} WT and KO mice. Allergen challenge significantly increased lipid peroxides in BAL fluid of both A_{2A} WT and KO sensitized mice with values of 0.644± 0.04 µmol/l (n=8, $p<0.05$) and 0.926± 0.06 µmol/l (n=8, $P<0.05$), respectively as compared to the A_{2A} WT and KO control mice with values of 0.471± 0.026 µmol/l (n=10) and 0.643± 0.047 µmol/l (n=10), respectively (Fig. 9A). Moreover, A_{2A} KO control and sensitized groups ($P<0.05$) further had significantly increased levels of lipid peroxides in BAL fluid as compared to the respective A_{2A} WT control and sensitized groups. On the contrary, allergen challenge significantly decreased total antioxidant capacity (TAC) in both A_{2A} WT and KO sensitized groups with values of 138.8± 8.91 µmol/l (n=10, $p<0.01$) and 88.10± 8.46 µmol/l (n=10, $p<0.01$), respectively as compared to the A_{2A} WT and KO control groups with values of 207.5± 10.25 µmol/l (n=10) and 170.1± 22.84 µmol/l (n=10), respectively (Fig. 9B). Moreover,

KO sensitized group further had significantly decreased TAC in BAL fluid as compared to WT sensitized group ($P < 0.05$).

DISCUSSION

The present study showed enhanced airway reactivity to methacholine and inflammation in A_{2A}WT and KO sensitized mice after allergen challenge. Real-time PCR and western blot data showed decreased expression of A_{2A}AR in WT sensitized group and absence of expression in KO groups. A_{2A}AR downregulation or absence was associated with a decrease in cAMP levels and an increase in NF-kB and phospho-IkBα activation after allergen challenge in the lung. Absence of the A_{2A}AR was also associated with increased expression of iNOS in the lung and BAL cells, 3-nitrotyrosine (3-NT) formation in BAL cells and nitric oxide (NO) generation in the lung that was further related to increased lipid peroxidation and decrease in the level of non-enzymatic antioxidants in BAL fluid, collectively confirming the presence of nitrosative stress. Moreover, a decrease or absence in A_{2A}AR expression after allergen challenge downregulated other adenosine receptors in the lungs of both A_{2A} WT and KO mice. All of these effects were most marked in A_{2A} KO sensitized mice thereby suggesting an important role for A_{2A}AR in the pathophysiology of asthma.

Biological functions of adenosine are mediated by G-protein coupled receptors, which have been cloned and pharmacologically identified. Four distinct adenosine receptors, namely A_1, A_{2A}, A_{2B} and A_3, have been cloned in different species. These receptors, presenting a similar sequence among subtypes represent an integral part of membrane proteins (14). Recent evidences suggest that adenosine A_{2A} receptors activate a protective mechanism thereby playing a critical role in the downregulation of inflammation and tissue damage (26, 36). Activation of adenosine A_{2A} receptor affects multiple aspects of the inflammatory processes, modulating leukocyte activation and

degranulation, oxidative species production, adhesion molecules expression, cytokines release and mast cells degranulation (24).

A_{2A}AR activation has been shown to elevate intracellular cAMP levels via interaction with G_s and stimulation of adenylyl cyclase (23). We have found decreased expression of A_{2A} ARs in sensitized WT with absence of detectable A_{2A}ARs transcripts in A_{2A}KO mice. This decrease in A_{2A}AR in WT and absence in KO sensitized animals was associated with a decrease in cAMP levels in the lung. A decrease of A_{2A}ARs in WT and absence in KO mice after allergen challenge further resulted in increased expression of IkBα and NF-kB. It has been demonstrated earlier that asthmatic inflammation is associated with increased nuclear factor kappa B (NF-kB) activity (6,18), which is a pleiotropic transcription factor involved in many pro-inflammatory effects. The increased activation of IkBα and NF-kB in our study could be due to a decrease in the levels of cAMP since increase in cAMP levels has been to shown to directly block NF-kB-induced transcription through cyclic AMP-responsive element binding protein (5, 38). Cyclic AMP also activates PKA which serves as a dual switch in the IkB complex enhancing transcription of NF-kB activity through p65 in the deficiency of cAMP, but inhibiting IkB phosphorylation upon interaction of PKA with cAMP-bound PKA regulatory subunits (5, 30).

Interestingly, the absence or decrease in the expression and translation of A_{2A}AR after allergen challenge in both knock-out and wild type sensitized mice respectively also caused down-regulation of other three adenosine receptors. It could be possibly due to the feedback inhibition of the other three adenosine receptors by A_{2A}AR to prevent unopposed inflammatory damage to the lung as the activation of these adenosine

receptors leads to airway inflammation and bronchoconstriction (12, 33, 34, 35). Therefore, from this standpoint, A_{2A}AR may function as a checkpoint for other adenosine receptors in the lung. Upregulation of A_1 AR in our rabbit model of asthma (see 34 for review) and downregulation in murine model of asthma in the present study could be due to the species differences (i.e. rabbit versus mice) in regard to bronchoconstriction and inflammation. Secondly, the discrepancy could also arise due to the use of different immunization and challenge protocols for these two species, i.e. allergic sensitization is done with dust mite just after birth in rabbits and repeated biweekly for a month and then monthly thereafter until the experiments are conducted; whereas in mice the entire protocol is completed within 15 days (11, 12, 33, 35).

A_{2A} ARs are present on most of the inflammatory cells (including neutrophils, mast cells, macrophages, eosinophils, platelets, and T-cells (27, 44) and therefore, could modulate inflammatory events in the airways. In these cells, activation of A_{2A} adenosine receptor is almost universally inhibitory (24) due to increased generation of cAMP. Thus, in the present study, increased migration of inflammatory cells such as eosinophils, lymphocytes and neutrophils into the airways induced by ragweed challenge seem to have been caused by the downregulation of A_{2A} AR expression in WT sensitized mice and absence of A_{2A} AR expression in KO sensitized mice. Some recent studies have also shown an attenuation of lung inflammation after activation of A_{2A}AR by using the A_{2A}AR agonist CGS 21680 in allergic asthma models (4, 13).

In this study, we found increased iNOS expression in lung and BAL cells with associated increases in 3-NT immunoreactivity in BAL cells and total nitrates and nitrites (NOx) levels in the lung of WT and KO sensitized mice after allergen challenge.

Increased nitric oxide production from iNOS could lead to the 3-NT formation through NF-kB pathway (10, 42), which seems to be activated in our study due to deficiency of A_{2A}ARs. Recently A_{2A}AR gene transfer has been shown to suppress the induction of iNOS in vitro through inhibition of NF-kB (43). The mechanism by which production of NO leads to oxidative nitration of tissue proteins could be due to the formation of peroxynitrite that is the principal agent responsible for 3-NT formation. Protein nitration detected as 3-nitrotyrosine (3-NT), is thought to be associated with alteration in the function of both regulatory and structural proteins in asthmatic airways (2). It has been further suggested that peroxidases, such as neutrophil myeloperoxidase and eosinophil peroxidase, are responsible for at least some 3-NT formation (9, 46) and these inflammatory cells have been found to be higher in A_{2A}AR deficient sensitized mice. This suggests that these cells may be responsible for generation of NO-related nitrosative stress in the absence or down regulation of A_{2A}AR.

Airway inflammation is a characteristic feature of asthma and likely contributes to airway hyper-responsiveness following allergen challenge (3). Infiltration of inflammatory cells into the airways may be responsible for the liberation of bronchoconstrictive mediators such as reactive oxygen/nitrogen species (22). In this study, sensitized A_{2A} KO mice had the highest airway reactivity following ragweed challenge, possibly due to absence of A_{2A}ARs on airway cells and subsequent generation of NO-related nitrosative stress. Peroxynitrite is thought to be responsible for the formation 3-NT, which has been shown to be correlated with expression of iNOS and inversely with hyperresponsiveness in asthma (42). Peroxynitrite has also been shown to cause increases in airway hyperresponsiveness, respiratory epithelial damage and

eosinophil activation in guinea pigs (41). The alveolar fluid, containing low and high molecular weight non-enzymatic antioxidants provides protection against nitrosative stress. Peroxynitrite is also known to cause extensive cell damage through lipid peroxidation and depletion of antioxidants (2, 39, 40). We found increased lipid peroxidation and decreased total antioxidant capacity, possibly due to the increased nitrosative stress caused due to the deletion or down regulation of A_{2A} AR. Lipid peroxidation and non-enzymatic antioxidants have also been linked to airway obstruction in our earlier studies on human asthmatics (31, 32). Therefore, these observations suggest that increased nitrosative stress caused due to deficiency of A_{2A}AR may be responsible for enhanced airway reactivity.

Use of inhibitors/activators of signaling pathways linking A_{2A}AR with nitrosative stress will further confirm our present findings, therefore will be explored in future experiments. Whole lung experiments though provide important information about overall pathophysiology, still the studies on the role of each AR in the individual cell type of the lung (e.g. airway smooth muscle, inflammatory cells and other cell types) will be useful to gain further insight into the complex pathophysiology of asthma.

The data presented in this study regarding AHR stands in contrast to those from a previous study whereby A_{2A}AR function was manipulated by the use of an A_{2A}AR agonist (4). Mice were treated prophylactically with the A_{2A}AR agonist before allergen challenge in sensitized mice. This treatment was found to reduce airway inflammation, but not airway hyper reactivity, which could be due to A_{2A} agonist-induced desensitization associated with phosphorylation of the receptor (37). Failure to inhibit AHR by A_{2A}AR agonist, CGS 21680 in their model could also be due to the non-specific

activation of A_3AR as suggested by authors themselves (4). A_3AR has been reported to cause bronchoconstriction in allergic asthma models (12). Therefore, the use of an agonist may be relatively less selective as opposed to the use of knock-out strategy in delineating the involvement of individual ARs.

In conclusion, we have demonstrated that deficiency of A_{2A}AR after allergen challenge results in increased NF-kB and IkBα activation leading to subsequent nitrosative stress. The ability to specifically inhibit NF-kB activation and nitrosative stress is likely the important reason for the inhibitory effects of A_{2A}AR on airway reactivity and inflammation. As such, our observations convincingly suggest the importance of the A_{2A}AR as both a key target for generation of anti-inflammatory strategies and a gene whose defective regulation may influence the pathophysiology of asthma. This further suggests that A_{2A} agonists could be developed as potential therapeutic strategy for the treatment of asthma.

GRANTS

This work is supported in part by National Heart, Lung and Blood Institute Grant HL-027339.

REFERENCES

1. **Allen-Gipson DS, Wong J, Spurzem JR, Sisson JH, and Wyatt TA.** Adenosine A_{2A} receptors promote adenosine-stimulated wound healing in bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 290: L849-L855, 2006.
2. **Andreadis AA, Hazen SL, Comhair SA, and Erzurum SC.** Oxidative and nitrosative events in asthma. *Free Radic Biol Med* 35:213-225, 2003.
3. **Barnes PJ, Fan Chung K, and Page CP.** Inflammatory mediators of asthma: an update. *Pharmacol Rev* 50: 515-596, 1998.
4. **Bonneau O, Wyss D, Ferretti S, Blaydon C, Stevenson CS, and Trifilieff A.** Effect of adenosine A_{2A} receptor activation in murine models of respiratory disorders. *Am J Physiol Lung Cell Mol Physiol* 290:L1036-L1043, 2006.
5. **Bshesh K, Zhao B, Spight D, Biaggioni I, Feokistov I, Denenberg A, Wong HR, and Shanley TP.** The A_{2A} receptor mediates an endogenous regulatory pathway of cytokine expression in THP-1 cells. *J Leukoc Biol* 72:1027-1036, 2002.
6. **Bureau F, Bonizzi G, Kirschvink N, Delhalle S, Desmecht D, Merville MP, Bours V, and Lekeux P.** Correlation between nuclear factor-κB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am J Respir Crit Care Med* 161:1314-1321, 2000.
7. **Busse WW, and Lemanske RF Jr.** Asthma. *N Engl J Med* 344:350-362, 2001.

8. **Driver AG, Kukoly CA, Ali S and Mustafa SJ.** Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 148: 91-97, 1993.
9. **Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, and van der Vliet A.** Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393-397, 1998.
10. **Escoubet-Lozach L, Glass CK, and Wasserman SI.** The role of transcription factors in allergic inflammation. *J Allergy Clin Immunol* 110:553-564, 2002.
11. **Fan M, and Mustafa SJ.** Adenosine-mediated bronchoconstriction and lung inflammation in an allergic mouse model. *Pul Pharmacol Ther* 15:147-155, 2002.
12. **Fan M, Qin W, and Mustafa SJ.** Characterization of adenosine receptor(s) involved in adenosine-induced bronchoconstriction in an allergic mouse model. *Am J Physiol Lung Cell Mol Physiol* 284:L1012-L1019, 2003.
13. **Fozard JR, Ellis KM, Villela Dantas MF, Tigani B, and Mazzoni L.** Effects of CGS 21680, a selective adenosine A_{2A} receptor agonist, on allergic airways inflammation in the rat. *Eur J Pharmacol* 438:183-188, 2002.
14. **Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, and Linden J.** International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 53:352–527, 2001.
15. **Ghiselli A, Serafini M, Natella F, and Scaccini N.** Total antioxidant capacity to assess redox status: critical view and experimental data. *Free Radic Biol Med* 29:1106-1114, 2000.

16. **Grisham MB, Johnson GG, and Lancaster JR.** Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* 268:237-246, 1996.

17. **Habib RA, Nadeem A, Talukder MAH, and Mustafa SJ.** Evidence for the involvement of nitric oxide in A_{2B} receptor-mediated vasorelaxation of mouse aorta. *Am J Physiol Heart Circul Physiol* 292:H 719-H 725, 2007.

18. **Hart LA, Krishnan VL, Adcock IM, Barnes PJ, and Chung KF.** Activation and localization of transcription factor, nuclear factor-κB, in asthma. *Am J Respir Crit Care Med* 158:1585-1592, 1998.

19. **Heffner JE, and Repine JE.** Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 140:531-554, 1989.

20. **Jentzsch AM, Bachmann H, Furst P, and Biesalski HK.** Improved analysis of malondialdehyde in human body fluids. *Free Radic Biol Med* 20:251-256, 1996.

21. **Justice, JP, Shibata Y, Sur S, Mustafa J, Fan M, and Van Scott MR.** IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am J Physiol Lung Cell Mol Physiol* 280: L363-L368, 2001.

22. **Katsumata MJ, Miura M, Ichinose M, Kimura K, Takahasi T, Inoue H, and Tikishima T.** Oxygen radicals produce airway constriction and hyperresponsiveness in anaesthetized cats. *Am Rev Respir Dis* 141:1158-1161, 1990.

23. **Klinger M, Kuhn M, Just H, Stefan E, Palmer T, Freissmuth M, and Nanoff C.** Removal of the carboxy terminus of the A_{2A}-adenosine receptor blunts constitutive activity: differential effect on cAMP accumulation and

MAP kinase stimulation. *Naunyn Schmiedeberg's Arch Pharmacol* 366: 287-298, 2002.

24. **Lappas CM, Sullivan GW, and Linden J.** Adenosine A_{2A} agonists in development for the treatment of inflammation. *Expert Opin Investig Drugs* 14:797-806, 2005.
25. **Livak KJ, and Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408, 2001.
26. **Lukashev D, Ohta A, Apasov S, Chen JF, and Sitkovsky M.** Cutting edge: Physiologic attenuation of proinflammatory transcription by the Gs protein-coupled A_{2A} adenosine receptor in vivo. *J Immunol* 173:21-24, 2004.
27. **Lukashev DE, Smith PT, Caldwell CC, Ohta A, Apasov SG, and Sitkovsky MV.** Analysis of A_{2A} receptor-deficient mice reveals no significant compensatory increases in the expression of A_{2B}, A_1, and A_3 adenosine receptors in lymphoid organs. *Biochem Pharmacol* 65:2081-2090, 2003.
28. **Marala RB, and Mustafa SJ.** Immunological characterization of adenosine A_{2A} receptors in human and porcine cardiovascular tissues. *J Pharmacol Exp Ther* 286:1051-1057, 1998.
29. **Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, and Milner A.** A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 84:407-412, 1993.

30. **Minguet S, Huber M, Rosenkranz L, Schamel WW, Reth M, and Brummer T.** Adenosine and cAMP are potent inhibitors of the NF-kappa B pathway downstream of immunoreceptors. *Eur J Immunol* 35:31-41, 2005.

31. **Nadeem A, Chhabra SK, Masood A, and Raj HG.** Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol* 111: 72-78, 2003.

32. **Nadeem A, Chhabra SK, and Raj HG.** Increased oxidative stress in acute exacerbations of asthma. *J Asthma* 42:45-50, 2005.

33. **Nadeem A, Obiefuna PC, Wilson CN, and Mustafa SJ.** Adenosine A(1) receptor antagonist versus montelukast on airway reactivity and inflammation. *Eur J Pharmacol* 551: 116- 124, 2006.

34. **Nadeem A, and Mustafa SJ.** Adenosine receptor antagonists and asthma. *Drug Disc Today: Therapeutic Strategies* 3: 269- 275, 2006.

35. **Obiefuna PC, Batra VK, Nadeem A, Borron P, Wilson CN, and Mustafa SJ.** A Novel A1 Adenosine Receptor Antagonist (L-97-1) Reduces Allergic Responses to House Dust Mite in an Allergic Rabbit Model of Asthma. *J Pharmacol Exp Ther* 315: 329-336, 2005.

36. **Ohta A, and Sitkovsky M.** Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916–920, 2001.

37. **Palmer, TM, Gettys TW, Jacobson KA, and Stiles GL.** Desensitization of the canine A_{2A} adenosine receptor: delineation of multiple processes. *Mol Pharmacol* 45:1082-1094, 1994.

38. **Parry GC, and Mackman N.** Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-κB-mediated transcription. *J Immunol* 159: 5450-5456, 1997.

39. **Radi R, Beckman JS, Bush KM, and Freeman BA**. Peroxynitrite oxidation of sulfhydrils. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266:4244-4250, 1991.

40. **Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanraman B, Barnes S, Kirk M, and Freeman BA.** Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. *J Biol Chem* 269:26066-26075, 1994.

41. **Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AKCP, Linde HG, Ark I, Coene A, and Nijkamp FP.** Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* 153:697–1701, 1996.

42. **Saleh D, Ernst P, Lim S, Barnes PJ, and Giaid A.** Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J* 12: 929-937, 1998.

43. **Sands WA, Martin AF, Strong EW, and Palmer TM.** Specific inhibition of nuclear factor-kappaB-dependent inflammatory responses by cell type-specific mechanisms upon A_{2A} adenosine receptor gene transfer. *Mol Pharmacol* 66:1147-1159, 2004.

44. **Thiel M, Caldwell CC, and Sitkovsky MV.** The critical role of adenosine A_{2A} receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. *Microbes Infect* 5:515-526, 2003.

45. **Visser SS, Theron AJ, Ramafi G, Ker JA, and Anderson R**. Apparent involvement of the $A_{(2A)}$subtype adenosine receptor in the anti-inflammatory interactions of CGS 21680, cyclopentyladenosine, and IB-MECA with human neutrophils. *Biochem Pharmacol* 60:993-999, 2000.

46. **Wu W, Chen Y, and Hazen SL.** Eosinophil peroxidase nitrates protein tyrosyl residues: implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem* 274: 25933-25944, 1999.

FIGURE LEGENDS

Fig. 1 Expression of all four adenosine receptors by real time PCR in lungs of A_{2A} KO and WT control mice. 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 mean ± SE, *n* = 5-8. ND = Not Detectable

Fig. 2 Expression of A_{2A} adenosine receptors by (A) real time PCR, and (B) western blot analyses respectively; and (C) cAMP levels in the lungs of A_{2A} KO and WT sensitized and control mice. For gene expression by comparative C_T method using real time PCR, first column was made as the calibrator against which other group was compared. A_{2A} AR gene and protein expression was undetected in A_{2A} KO mice. The western blot is from one experiment that is a representative of three independent experiments. Values are expressed as mean ± SE, *n* = 5-7/group. * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ in comparison with the respective controls.

Fig. 3 Expression of (A) A_1, (B) A_{2B} and (C) A_3 adenosine receptors by real time PCR in lungs of A_{2A} KO and WT sensitized and control mice. 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 mean ± SE, *n* = 5-8/group. ** $P < 0.01$ in comparison with the respective controls; $^{\#} P < 0.05$ in comparison with the respective WT mice.

Fig. 4 Airway inflammation in BAL one day after final allergen/saline challenge in A_{2A} KO and WT sensitized and control mice. (A) Total cells and differential cell counts for (B) eosinophils; (C) lymphocytes; and (D) neutrophils in BAL from KO and WT control and sensitized mice. Values are expressed as mean ± SE, n = 8-10/group. * $P < 0.05$ in comparison with the respective controls; $^{\#}P < 0.05$ in comparison with the respective WT mice.

Fig. 5 Airway reactivity in A_{2A} KO and WT sensitized and control mice. Airway reactivity to methacholine was measured 24 h after the final allergen challenge using a Buxco system for whole body plethysmography in which mice were exposed to increasing concentrations of methacholine (3-25 mg/ml). Values are expressed as mean ± SE, n = 8-10/group. * $P < 0.05$ in comparison with the respective controls; $^{\#}P < 0.05$ in comparison with the respective WT mice.

Fig. 6 Expression of (A) p-65 subunit of NF-kB and (B) phospho-IkBα by real-time PCR and western blot respectively in the lungs of A_{2A} KO and WT sensitized and control mice. 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. The western blot is from one experiment that is a representative of three independent experiments. Values are expressed as mean ± SE, n = 5-6/group. * $P < 0.05$ & ** $P < 0.01$ in comparison with the respective controls; $^{\#}P < 0.05$ in comparison with the respective WT mice.

Fig. 7 (A) iNOS gene expression and (B) total nitrates and nitrites (NOx) in the lungs of A_{2A} KO and WT sensitized and control mice. 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 mean ± SE, n = 5/group. * P <0.05 & ** P < 0.01 in comparison with the respective controls; $^{\#\#}P$ <0.05 in comparison with the respective WT mice.

Fig. 8 iNOS and 3-NT immunoreactivity in BAL cells of A_{2A} KO and WT sensitized and control mice. Representative immunofluorescent photomicrographs of iNOS (A & B; two photos in the top panel; orange staining) and 3-NT immunostaing (C & D; two photos in the bottom panel; green staining) from A_{2A} WT and KO allergen sensitized and challenged mice (magnification x200). Control mice in both groups showed almost negligible staining for iNOS and 3-NT (n = 3-4/group).

Fig. 9 (A) Lipid peroxidation, and (B) total antioxidant capacity in BAL fluid of A_{2A} KO and WT sensitized and control mice. Values are expressed as mean ± SE, n = 8-10/group. * P < 0.05 & ** P < 0.01 in comparison with the respective controls; $^{\#}P$ <0.05 in comparison with the respective WT mice.

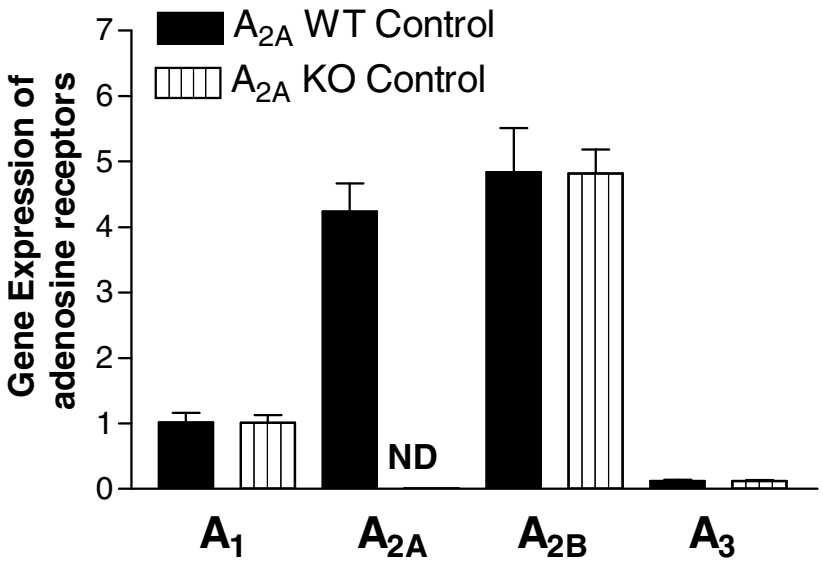
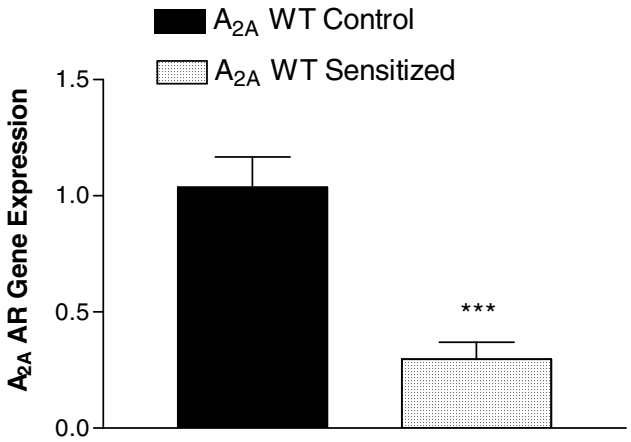
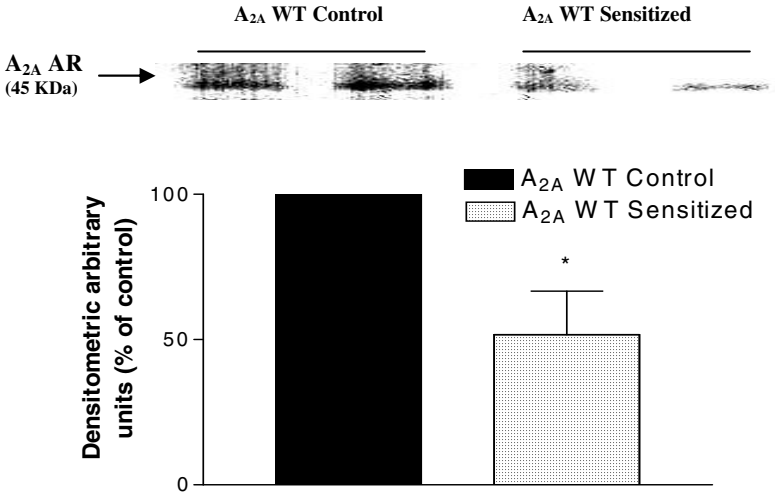

A2A WT Control
A2A KO Control
Gene Expression of adenosine receptors
7
6
5
4
3
2
1
0
ND
A1
A2A
A2B
A3

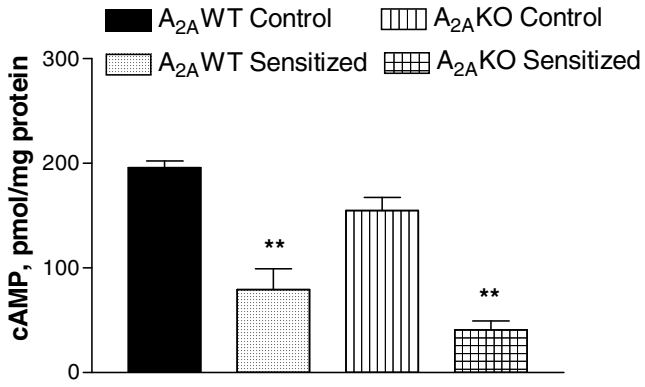
Fig. 1


A2A WT Control
A2A WT Sensitized
A2A AR Gene Expression
1.5
1.0
0.5
0.0
***

(A)

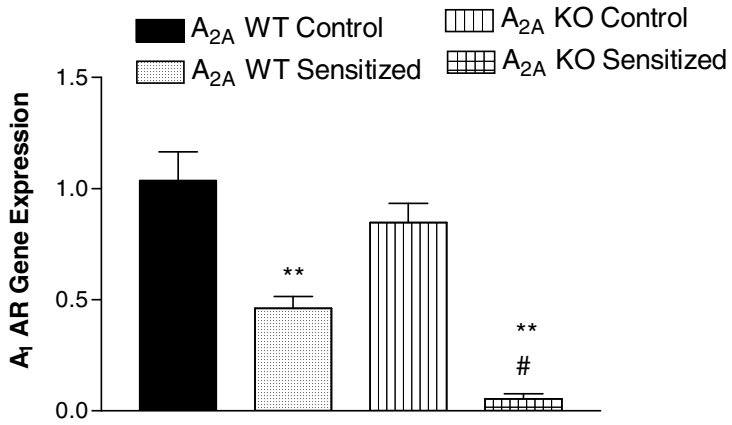

A2A WT Control
A2A WT Sensitized
A2A AR
(45 KDa)
A2A WT Control
A2A WT Sensitized
Densitometric arbitrary units (% of control)
100
50
0
*

(B)

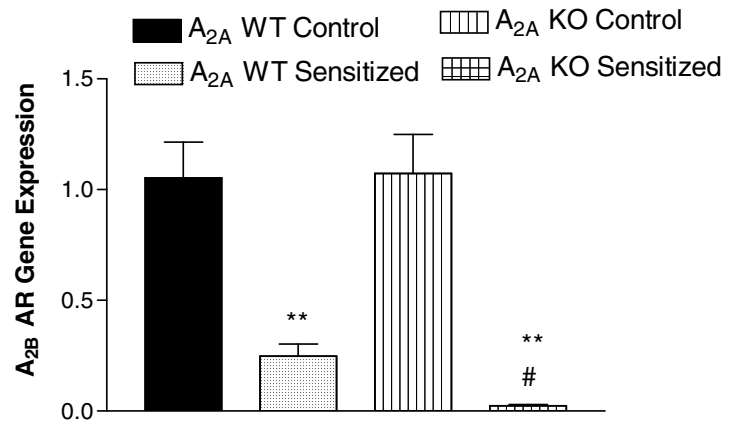

A2AWT Control
A2AKO Control
A2AWT Sensitized
A2AKO Sensitized
cAMP, pmol/mg protein
300
200
100
0
**
**

(C)

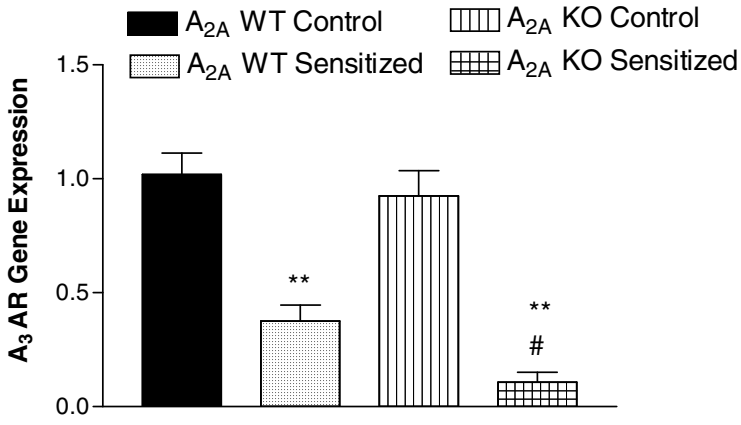
Fig. 2


A2A WT Control
A2A KO Control
A2A WT Sensitized
A2A KO Sensitized
A1 AR Gene Expression
1.5
1.0
0.5
0.0
**
**
#

(A)


A2A WT Control
A2A KO Control
A2A WT Sensitized
A2A KO Sensitized
A2B AR Gene Expression
1.5
1.0
0.5
0.0
**
**
#

(B)


A2A WT Control
A2A KO Control
A2A WT Sensitized
A2A KO Sensitized
A3 AR Gene Expression
1.5
1.0
0.5
0.0
**
**
#

(C)

Fig. 3

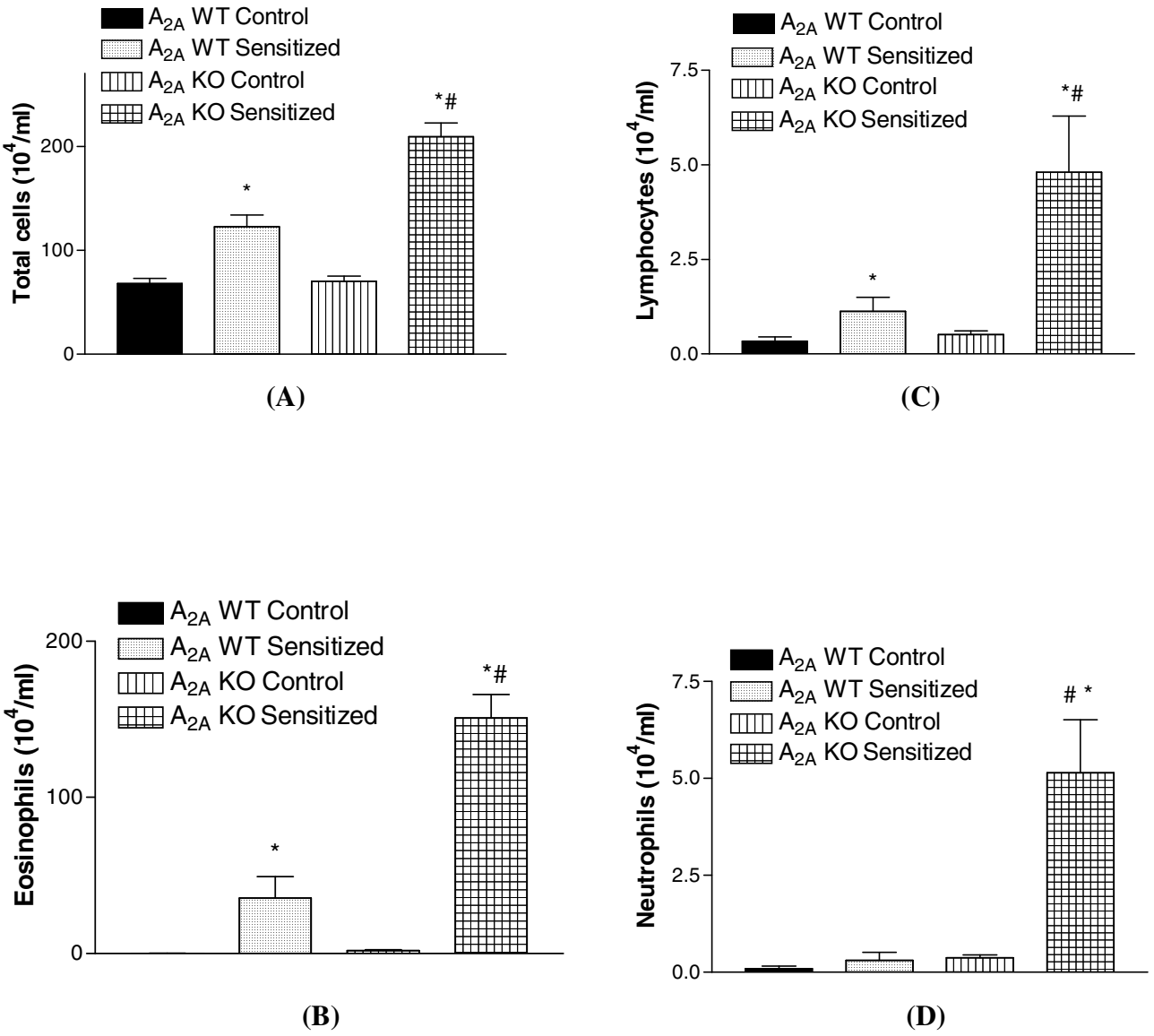

A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
Total cells (10^4/ml)
Lymphocytes (10^4/ml)
Eosinophils (10^4/ml)
Neutrophils (10^4/ml)
(A)
(C)
(B)
(D)

Fig. 4

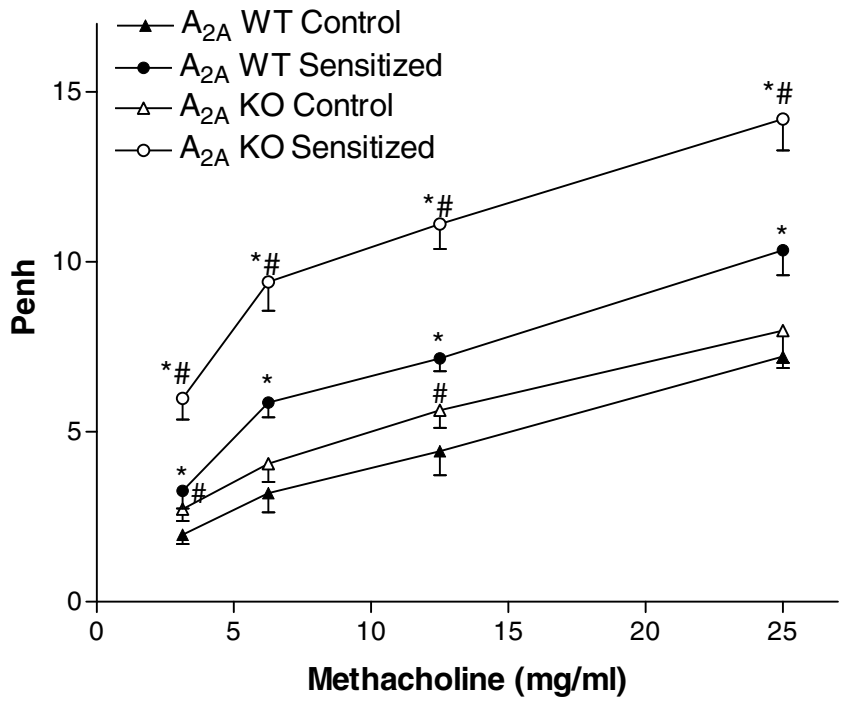
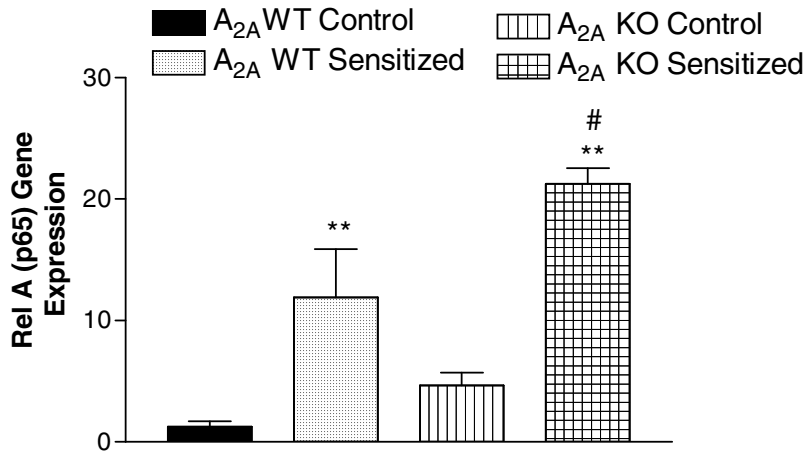
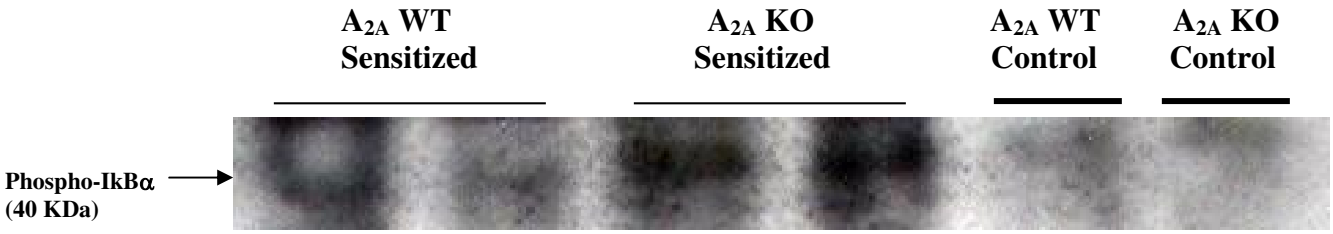
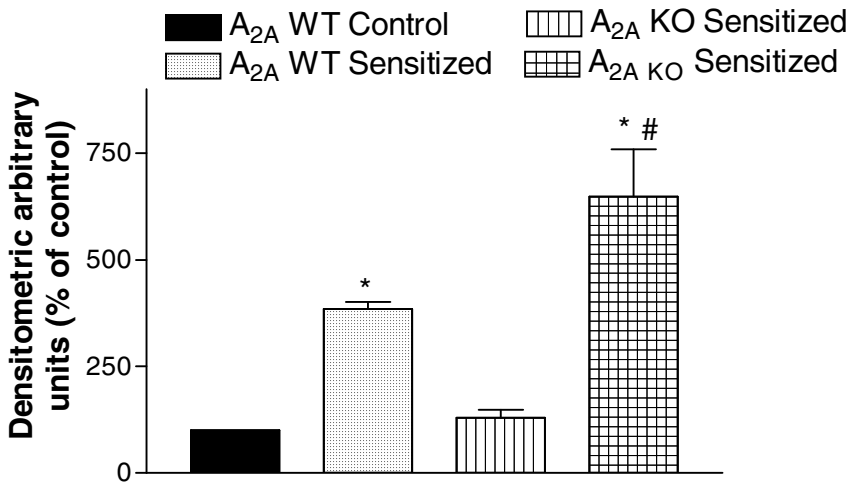

A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
Penh
Methacholine (mg/ml)
0
5
10
15
20
25

Fig. 5


A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
Rel A (p65) Gene Expression
30
20
10
0
**
**
#

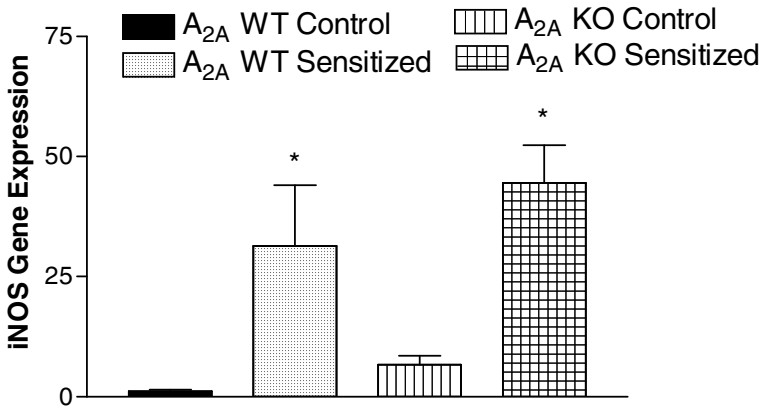
(A)


A2A WT Sensitized
A2A KO Sensitized
A2A WT Control
A2A KO Control
Phospho-IkBα
(40 KDa)

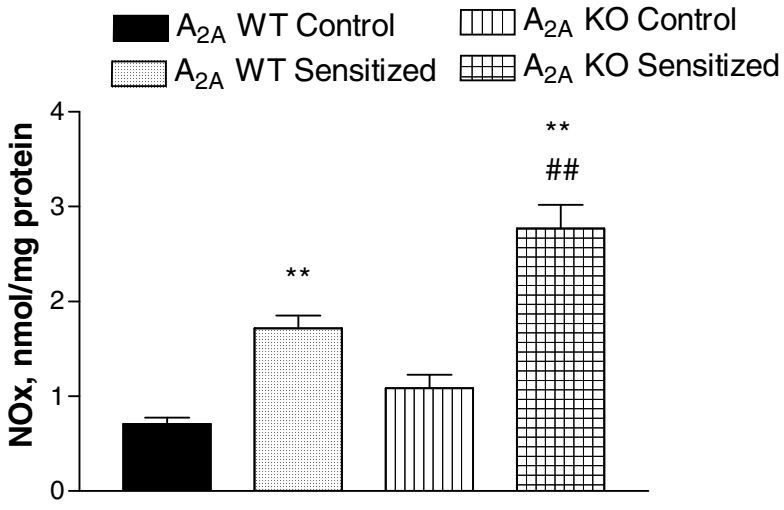

A2A WT Control
A2A WT Sensitized
A2A KO Sensitized
A2A KO Sensitized
Densitometric arbitrary units (% of control)
750
500
250
0
*
* #

(B)

Fig. 6


A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
iNOS Gene Expression
75
50
25
0
*
*

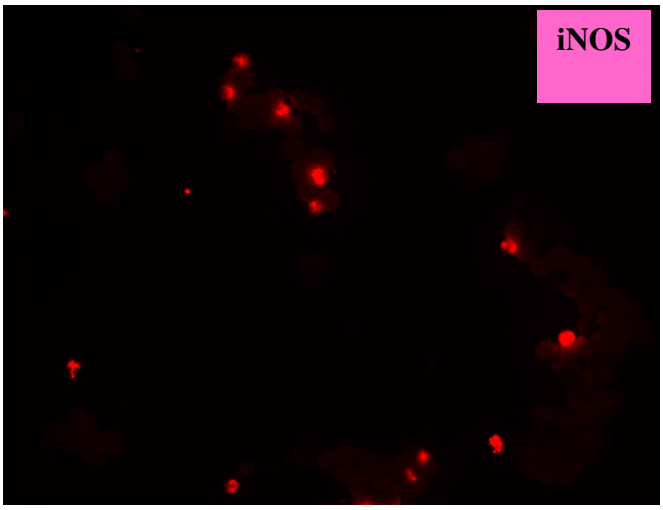
(A)


A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
NOx, nmol/mg protein
4
3
2
1
0
**
**
##

(B)

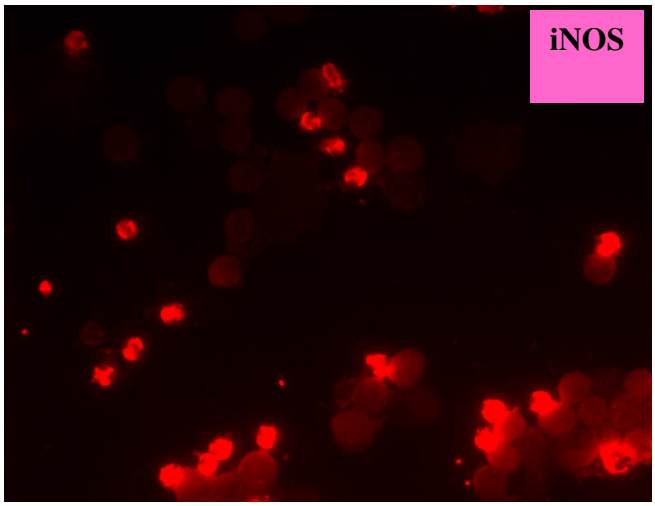
Fig. 7

A_{2A} WT Sensitized

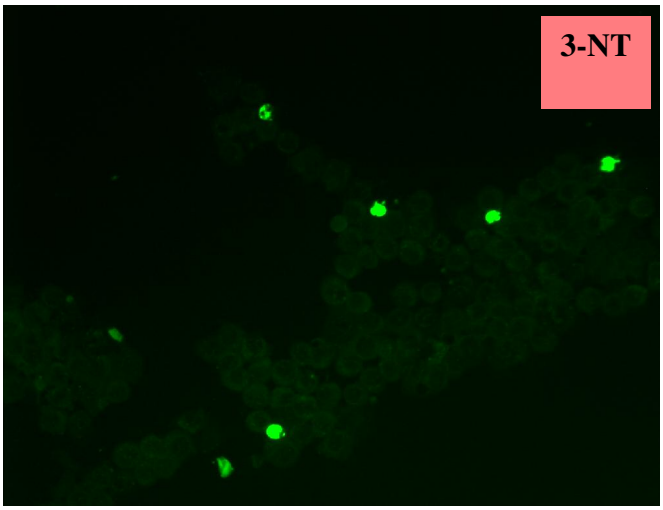


(A)

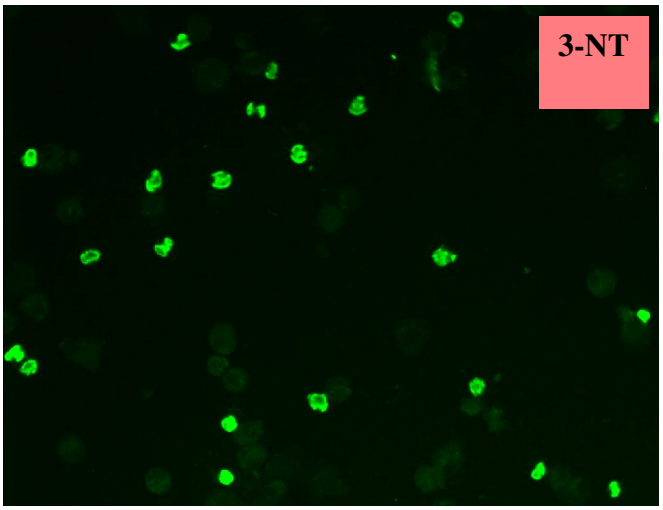
A_{2A} KO Sensitized



(B)

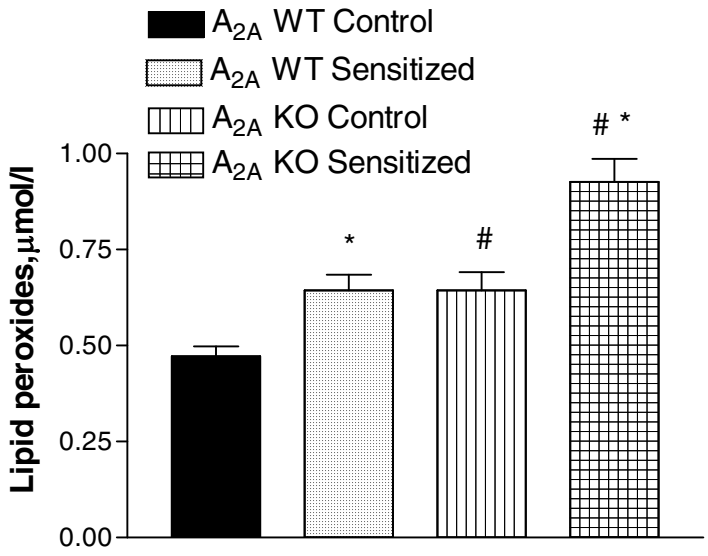


(C)

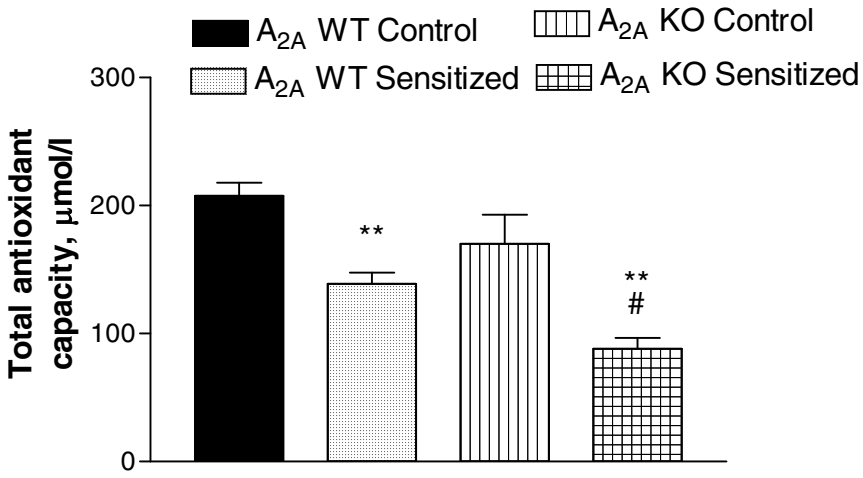


(D)

Fig. 8


A2A WT Control
A2A WT Sensitized
A2A KO Control
A2A KO Sensitized
Lipid peroxides,µmol/l
1.00
0.75
0.50
0.25
0.00
*
#
*

(A)


A2A WT Control
A2A KO Control
A2A WT Sensitized
A2A KO Sensitized
Total antioxidant capacity, µmol/l
300
200
100
0
**
**
#

(B)

Fig. 9