Enhanced airway reactivity and inflammation in A2A adenosine receptor-deficient allergic mice

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1Department of Physiology and Pharmacology, School of Medicine, West Virginia University, Morgantown, West Virginia; 2Department of Pharmacology, East Carolina University, Greenville, North Carolina; and 4Institut de Recherche Interdisciplinaire en Biologie Humaine et Nucleaire, Universite Libre de Bruxelles, Brussels, Belgium

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Nadeem A, Fan M, Ansari HR, Ledent C, Mustafa SJ. Enhanced airway reactivity and inflammation in A2A adenosine receptor-deficient allergic mice. Am J Physiol Lung Cell Mol Physiol 292: L1335–L1344, 2007. First published February 9, 2007; doi:10.1152/ajplung.00416.2006.—A2A adenosine receptor (A2AAR) has potent anti-inflammatory properties, which may be important in the regulation of airway reactivity and inflammation. Inflammatory cells that possess A2AAR also produce nitrosative stress, which is associated with pathophysiology of asthma, so we hypothesized that A2AAR deficiency may lead to increased airway reactivity and inflammation through nitrosative stress. Thus the present study was carried out to investigate the role of A2AAR on airway reactivity, inflammation, NF-κB signaling, and nitrosative stress in A2AAR knockout (KO) and wild-type (WT) mice using our murine model of asthma. Animals were sensitized intraperitoneally on days 1 and 6 with 200 μg 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 a significant decrease in expression of A2AAR in A2A WT sensitized mice, with A2AAR expression being undetected in A2A KO sensitized group leading to decreased lung cAMP levels in both groups. A2AAR 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 A2A KO sensitized group. A2A KO sensitized group further had increased NF-κB expression and nitrosative stress compared with WT sensitized group. These data suggest that A2AAR deficiency leads to airway inflammation and airway hyperresponsiveness, possibly via involvement of nitrosative stress in this model of asthma.

NF-κB signaling

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 A1, A2A, A2B, and A3, have been identified. Each receptor has unique tissue distribution, ligand affinity, and signal transduction pathways (14).

Strong anti-inflammatory properties for A2A adenosine receptors (A2AAR) have recently been described in various systems (26, 36). The A2AAR is expressed on inflammatory cells, including neutrophils, mast cells, lymphocytes, macrophages, and monocytes (27, 44). Activation of A2AAR causes reduction in chemotaxis, phagocytosis, the adherence of neutrophils to endothelial cells, and the secretion of proinflammatory 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 A2AAR causes its pathophysiological actions in asthma. Recently, the activation of A2AARs has also been shown to exert inhibitory effects on NF-κB signaling in vitro (5, 30, 43).

Inflammatory cells possess A2AARs (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 A2AARs is mediated through attenuation of nitrosative stress, possibly via suppression of inducible nitric oxide synthase (iNOS) expression and 3-nitrotyrosine (3-NT) formation. A2AAR 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 A2AAR can lead to depletion of endogenous nonenzymatic 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 A2AARs on airway reactivity, inflammation, and nitrosative stress using A2AAR knockout (KO) mice.

MATERIALS AND METHODS

Mice Sensitization and Challenge

A2A KO and wild-type (WT) mice were obtained from the Institute of Experimental Medicine (C. Ledent, Universite Libre de Bruxelles, Brussels, Belgium).
Brussels, Belgium). To homogenize the genetic background of the mice, the first-generation heterozygotes were bred for 14 generations on a CD1 (Charles River) outbred background, with selection for the mutant A2A gene at each generation by PCR. Fourteenth-generation heterozygotes were bred together to generate the A2A KO and WT.

Male A2A KO and their WT littermate controls, 11–14 wk 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 intraperitoneal injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 μg per dose with 200 μl Imject alum (Pierce Laboratories, 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 0.5% aerosolized ragweed or with 0.9% saline as a control, using an ultrasonic nebulizer (DeVilbiss, Somerset, PA) for 20 min both in the morning and in the afternoon for 3 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 (DeVilbiss).

Mice were divided into following groups: WT control group (A2A WT control) in which mice received only vehicles for sensitization and challenge; WT sensitized group (A2A WT sensitized) in which mice were sensitized and challenged with ragweed using the same protocol described above; KO control group (A2A KO control) in which mice received only vehicle for sensitization and challenge; and KO sensitized group (A2A KO sensitized) in which mice were sensi-
tized 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 methacholine in conscious, unrestrained mice was 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 Penh values were recorded and averaged for 5 min following each nebulization.

**Bronchoalveolar Lavage**

Mice were killed by intraperitoneal injection (0.1 ml pentobarbitone sodium, 200 mg/ml). The trachea was cannulated to perform bronchoalveolar lavage (BAL); 0.6 ml of 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 (75–80% 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). Approximately 1–5 × 10⁵ 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 morphological criteria. The number of cells recovered per mouse was calculated and expressed as means ± 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 of 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, pH 7.4, 1% Triton X-100 (Tx), 0.25% sodium deoxycholate, 1 mmol/l sodium chloride, 1 mmol/l EDTA, 1 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, 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 of BAL fluid. This kit measures mainly nonenzymatic antioxidants in 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 hydroxytoluene, 0.2 mol/l orthophosphoric acid, and 0.11 M thiobarbituric acid (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 multiter plate reader. MDA-TBA adducts were calculated using the difference in absorption at the two wavelengths compared with the standard curve generated by the use of tetraethoxypropane. Results were expressed in μmol/l.

**NOx assay.** NO production in lung homogenate supernatant was detected as NOx by the method of Grisham et al. (16), which are the end products of NO metabolism and provide one of the most useful methods to quantify NO 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 1 M zinc sulfate to precipitate the proteins. After centrifugation, 50 μl of supernatant from each microtube was transferred into individual wells of a 96-well microplate, followed by addition of 100 μl of Griess reagent [1:1 mixture of 1% (wt/vol) sulfanilamide in 5% (vol/vol) orthophosphoric acid and 0.1% (wt/vol) 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-NT and iNOS**

After 5 min of washing in PBS-Tx + 1% bovine serum albumin (PBS-Tx-BSA, pH 7.8), defrosted coverslips with BAL cells were incubated with an anti-3-NT 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 three times with PBS-Tx-BSA, allowing 5 min per rinse, and then covered with FITC-labeled goat anti-rabbit IgG (Zymed, San Francisco, CA) for 3-NT and rhodamine-conjugated goat anti-rabbit IgG (Upstate) for iNOS diluted 1:100 in PBS-Tx-BSA and incubated at 37°C for 45 min. After that, the coverslips were rinsed three 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 and absence of staining with preincubation with 500 mmol/l sodium hydrosulfite (dithionite) dissolved in 100 mmol/l sodium borate (Sigma) that reduces 3-NT to aminotyrosine.

**Western Blot for A2AAR and Phosphorylated IκBα**

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-IκBα mouse monoclonal IgG (Cell Signaling Technology, Danvers, MA) for detection of active form of IκBα or anti-A2AAR polyclonal rabbit IgG (28) developed in our laboratory for detection of A2AAR expression diluted 1:1,000, followed by an incubation with...
the secondary HRP-conjugated antibodies (anti-mouse and anti-rabbit immunoglobulins from goat for phospho-IκBα and A2AR, respectively; Amersham Pharmacia Biotech) for 1 h at RT. 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, San Leandro, CA). Western blot values are expressed as % of control after densitometric analysis.

Real-Time PCR for AR, iNOS, and p-65 Subunit of NF-κB

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 manufacturer’s instructions 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, 1 μl of 2× Taqman Universal Mastermix, 1 μl of cDNA, and 1.25 μl of 20× FAM-labeled Taqman gene expression assay master mix solution. For real-time PCR for all four ARs, iNOS, and p-65 subunit of NF-κB genes, the Taqman inventoried assays-on-demand gene expression product with GenBank acc. nos. NM_001008533 (A1AR), NM_009630 (A2AR), NM_007413 (A2BAR), NM_009631 (A3AR), NM_010927 (iNOS), and NM_009045 (p-65) were purchased from Applied Biosystems. 18S ribosomal RNA (GenBank acc. no. X03205) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative CT method (25). The ΔCT value was determined in each group by subtracting the average 18S CT value from the corresponding average CT for each gene. The ΔΔCT of each gene in different groups was calculated by subtracting ΔCT of the first group (1st column in each graph) by ΔCT of the other groups. The fold difference in gene expression of the target was calculated as the average value from 2ΔΔCT+s and 2−ΔΔCT−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 Chemical (St. Louis, MO).

Statistical Analysis

The data were expressed as means ± SE. Comparisons among different groups were analyzed by ANOVA 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 GraphPad Prism statistical package.

RESULTS

Expression of ARs and cAMP Levels in the Lung After Allergen Challenge in A2A WT and KO Mice

As shown in Fig. 1, expression of all four ARs was found in the lung with A2AR and A2BAR having the highest expression and A3AR having the lowest expression in the A2A WT control mice. A similar pattern was also found in the A2A WT control mice except that no transcripts were detected for A2AR gene (Fig. 1). Allergen challenge significantly decreased the expression of A2ARs by 3.5-fold in A2A WT sensitized mice (P < 0.001) compared with A2A WT control mice, whereas there was undetectable expression of these receptors in the A2A KO mice (Fig. 2A). This decreased gene expression was further confirmed by Western blot, which showed almost 50% less protein expression for A2AR in A2A WT sensitized group compared with A2A WT control group (P < 0.05; Fig. 2B). Decreased expression of A2ARs in WT sensitized mice and deletion of A2ARs in KO sensitized mice was associated with 60% and 74% decrease in cAMP levels compared with the A2A WT and KO control mice, respectively (Fig. 2C; P < 0.01). Additionally, A2A KO sensitized mice had a further decrease of 48% in cAMP levels compared with the A2A WT sensitized mice.

Allergen challenge also significantly decreased the expression of A1 (Fig. 3A), A2B (Fig. 3B), and A3 ARs (Fig. 3C) in A2A WT (P < 0.01) and KO (P < 0.01) sensitized mice compared with the respective A2A WT and KO control mice. In these groups, the A2A KO sensitized group had the least expression of A1, A2B, and A3 ARs, which were also significantly different from A2A WT sensitized group (Fig. 3, A–C; P < 0.05).

Leukocyte Recruitment to the Airways After Allergen Challenge in A2A 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 A2A WT (80%; P < 0.05) and A2A KO (190%; P < 0.05) sensitized groups after aerosolized ragweed challenge compared with
their respective A2A WT and KO control mice. A2A KO sensitized mice further had an increase of 70% (P < 0.05) in total cells compared with corresponding WT mice (Fig. 4A).

Out of the total cells recovered from BAL, eosinophils were the major cell consisting of 29% and 72% in A2A WT and KO sensitized mice, respectively. Eosinophils (Fig. 4B) and lymphocytes (Fig. 4C) increased significantly in both A2A WT (P < 0.05) and KO (P < 0.05) sensitized groups compared with their respective A2A WT and KO control mice. A2A KO sensitized group (P < 0.05) further had significant difference in both eosinophils and lymphocytes compared with A2A WT sensitized group. Increase in the number of neutrophils was found only in A2A KO sensitized mice, which was significantly different from both A2A KO control (P < 0.05) and A2A WT sensitized (P < 0.05) groups (Fig. 4D). On the other hand, there was a slight decrease in the number of macrophages in A2A KO sensitized mice (P < 0.05) compared with A2A WT sensitized mice (data not shown).

**Airway Reactivity to Methacholine After Allergen Challenge in A2A WT and KO Mice**

To determine whether deficiency in A2AAR 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 A2A WT and KO mice. Figure 5 shows that there was a significant difference in response to methacholine even in A2A KO control compared with A2A WT control mice at doses of 3.125 and 12.5 mg/ml (P < 0.05). Both A2A WT and KO sensitized mice showed increased Penh following methacholine challenge at all doses compared with their respective A2A WT and KO control mice (P < 0.05). A significant increase in Penh following methacholine challenge was further observed in A2A KO sensitized mice compared with WT sensitized mice at all doses (Fig. 5; P < 0.05).

**Expression of Phosphorylated IκBα and p-65 subunit of NF-κB After Allergen Challenge in A2A WT and KO Mice**

Since cAMP is mostly responsible for anti-inflammatory effects of A2A AR, we expected that decreased cAMP levels caused by downregulation/deletion of A2A ARs might activate NF-κB. In accordance with this, real-time PCR analysis showed 9.7- and 4.6-fold higher expression of p-65 subunit of NF-κB in A2A WT and KO sensitized mice compared with the A2A WT and KO control mice, respectively (Fig. 6A; P < 0.01). Furthermore, the A2A KO sensitized group had almost twofold higher expression of p-65 compared with the A2A WT sensitized group (Fig. 6A; P < 0.05). The most important step in the regulation of NF-κB activity is its nuclear translocation following the release from its inhibitor IκBα, which is achieved by its phosphorylation and subsequent degradation. The experiment in Fig. 6B shows significantly higher phos-
phorylation of IκBα in A2AWT and KO sensitized groups (P < 0.05) compared with the respective A2A WT and KO control groups. A2A KO sensitized mice further had significant difference in expression compared with A2A WT sensitized mice (P < 0.05).

Expression of iNOS, 3-NT, and NOx in the Lung and BAL Cells After Allergen Challenge in A2A WT and KO Mice

NF-κB is thought to be a major transcriptional factor responsible for the upregulation of iNOS. Therefore, downstream effects of NF-κB 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 NO production as NOx in the lung. Real-time PCR showed that there was an increased expression of iNOS gene in the lung of A2A WT (P < 0.05) and KO (P < 0.05) sensitized mice compared with the A2A WT and KO control mice after allergen challenge (Fig. 7A). A2A KO sensitized group further had an increase of 42% in iNOS expression compared with A2A WT sensitized group (Fig. 7A). Increase in iNOS expression was associated with increased levels of lung NOx in both A2A 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, compared with the A2A WT (0.707 ± 0.068 nmol/mg protein, n = 5) and KO (1.085 ± 0.144 nmol/mg protein, n = 5) control mice. Moreover, A2A KO sensitized mice further had increased levels of lung NOx compared with the A2A 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 immunostaining with both anti-iNOS (Fig. 8, A and B) and 3-NT (Fig. 8, C and D) antibodies in A2A WT and KO sensitized groups. The immunostaining was seen much more in KO sensitized BAL cells for both iNOS and 3-NT compared with WT sensitized BAL cells. Most of these cells showed 3-NT immunostaining 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 3-NT 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 A2A WT and KO Mice

Lipid peroxidation and total antioxidant capacity in BAL fluid as markers of nitrosative stress were assessed in A2A WT and KO mice. Allergen challenge significantly increased lipid peroxides in BAL fluid of both A2A 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, compared with the A2A 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, A2A KO control and sensitized groups (P < 0.05) further had significantly increased levels of lipid peroxides in BAL fluid compared with the respective A2A WT control and sensitized groups. On the contrary, allergen challenge significantly decreased TAC in both A2A 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, compared with the A2A 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, the KO sensitized group further had significantly decreased TAC in BAL fluid compared with the WT sensitized group (P < 0.05; Fig. 9B).

DISCUSSION

The present study showed enhanced airway reactivity to methacholine and inflammation in A2A WT and KO sensitized mice after allergen challenge. Real-time PCR and Western blot data showed decreased expression of A2AAR in WT sensitized group and absence of expression in KO groups. A2AAR downregulation or absence was associated with a decrease in cAMP levels and an increase in NF-κB and phospho-IκBα activation after allergen challenge in the lung. Absence of the A2AAR was also associated with increased expression of iNOS in the lung and BAL cells, 3-NT formation in BAL cells, and NO generation in the lung that was further related to increased lipid peroxidation and a decrease in the level of nonenzymatic antioxidants in BAL fluid, collectively confirming the presence of nitrosative stress. Moreover, a decrease or absence in A2AAR expression after allergen challenge downregulated other ARs in the lungs of both A2A WT and KO mice. All of these effects were most marked in A2A KO sensitized mice, thereby suggesting an important role for A2AAR 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 ARs, namely A1, A2A, A2B, and A3, have been cloned in different species. These receptors, presenting a similar sequence among subtypes, represent an integral part of membrane proteins (14). Recent evidence suggests that A2AARs activate a protective mechanism, thereby playing a critical role in the downregulation of inflammation and tissue damage (26, 36). Activation of A2AAR affects multiple aspects of the inflammatory processes, modulating leukocyte activation and degranulation, oxidative species production, adhesion molecule expression, cytokine release, and mast cell degranulation (24).

A2AAR activation has been shown to elevate intracellular cAMP levels via interaction with Gs and stimulation of adenylyl cyclase (23). We have found decreased expression of A2AARs in sensitized WT with absence of detectable A2AAR transcripts in A2A KO mice. This decrease in A2AARs in WT and absence in KO sensitized animals was associated with a decrease in cAMP levels in the lung. A decrease of A2AARs in WT and absence in KO mice after allergen challenge further resulted in increased expression of IkBα and NF-κB. It was demonstrated earlier that asthmatic inflammation is associated with increased NF-κB activity (6, 18), which is a pleiotropic transcription factor involved in many proinflammatory effects. The increased activation of IkBα and NF-κB in our study could be due to a decrease in the levels of cAMP since increase in cAMP levels has been shown to directly block NF-κB-induced transcription through cAMP-responsive element binding protein (5, 38). cAMP also activates PKA, which serves as a dual switch in the IkB complex, enhancing transcription of NF-κB 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 A2AAR after allergen challenge in both KO and WT sensitized mice, respectively, also caused downregulation of three other ARs. It could be due to the feedback inhibition of the other three ARs by A2AAR to prevent unopposed inflammatory damage to the lung as the activation of these ARs leads to airway inflammation and bronchoconstriction (12, 33, 34, 35). Therefore, from this standpoint, A2AAR may function as a checkpoint for other ARs in the lung. Upregulation of A1AR in our rabbit model of asthma (see Ref. 34 for review) and downregulation in murine model of asthma in the present study could be due to the species differences (i.e., rabbit vs. mice) in regard to bronchoconstriction and inflammation. Second, 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).

A2AARs 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 A2AAR 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 A2AAR expression in WT sensitized mice and absence of

Fig. 8. iNOS and 3-nitrotyrosine (3-NT) immunoreactivity in BAL cells of A2A KO and WT sensitized and control mice. Representative immunofluorescent photomicrographs of iNOS (A and B; orange staining) and 3-NT immunostaining (C and D; green staining) from A2A WT and KO allergen sensitized and challenged mice (magnification, ×200). Control mice in both groups showed almost negligible staining for iNOS and 3-NT (n = 3–4/group).
A2AAR expression in KO sensitized mice. Some recent studies have also shown an attenuation of lung inflammation after activation of A2A AR by using the A2A 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 NOx levels in the lung of WT and KO sensitized mice after allergen challenge. Increased NO production from iNOS could lead to the 3-NT formation through NF-κB pathway (10, 42), which seems to be activated in our study due to deficiency of A2A ARs. Recently, A2A AR gene transfer has been shown to suppress the induction of iNOS in vitro through inhibition of NF-κB (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-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 A2A AR-deficient sensitized mice. This suggests that these cells may be responsible for generation of NO-related nitrosative stress in the absence or downregulation of A2A AR.

Airway inflammation is a characteristic feature of asthma and likely contributes to AHR 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 A2A KO mice had the highest airway reactivity following ragweed challenge, possibly due to absence of A2A 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 AHR, respiratory epithelial damage, and eosinophil activation in guinea pigs (41). The alveolar fluid, containing low- and high-molecular-weight nonenzymatic 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 TAC, possibly due to the increased nitrosative stress caused due to the deletion or downregulation of A2A AR. Lipid peroxidation and nonenzymatic 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 A2A AR may be responsible for enhanced airway reactivity.

The use of inhibitors/activators of signaling pathways linking A2A AR with nitrosative stress will further confirm our present findings and therefore will be explored in future experiments. Although whole lung experiments provide important information about overall pathophysiology, 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 A2A AR function was manipulated by the use of an A2A AR agonist (4). Mice were treated prophylactically with the A2A AR agonist before allergen challenge in sensitized mice. This treatment was found to reduce airway inflammation, but not airway hyperreactivity, which could be due to A2A agonist-induced desensitization associated with phosphorylation of the receptor (37). Failure to inhibit AHR by A2A AR agonist CGS-21680 in their model could also be due to the nonspecific activation of A3 AR, as suggested by the authors themselves (4). A3 AR 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 KO strategy in delineating the involvement of individual ARs.

In conclusion, we have demonstrated that deficiency of A2A AR after allergen challenge results in increased NF-κB and IκBα activation leading to subsequent nitrosative stress. The ability to specifically inhibit NF-κB activation and nitrosative stress is likely the important reason for the inhibitory effects of A2A AR on airway reactivity and inflammation. As such, our observations convincingly suggest the importance of the A2A 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 A2A AR agonists could be developed as potential therapeutic strategy for the treatment of asthma.
REFERENCES


