A₃ Adenosine Receptor Signaling Contributes to Airway Mucin Secretion after Allergen Challenge

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Mucin hypersecretion is a prominent feature of obstructive airway diseases such as asthma. Clara cells conditionally produce mucin in response to inflammatory signals in a process termed mucous metaplasia. This can be followed by mucin secretion stimulated by various signaling molecules. The cellular and molecular mechanisms that regulate mucin production and secretion are not well understood. Adenosine is a signaling nucleoside that has been implicated in airway diseases in which mucus obstruction is prominent. Furthermore, the A₃ adenosine receptor (A₃AR) is upregulated in mucin-producing goblet cells of the airway, thereby implicating it in processes involved in mucous cell biology. Here we use genetic approaches to investigate the contribution of A₃AR signaling to mucus production and secretion in a mouse model of allergen-induced pulmonary disease. We found that the degree of mucin production in response to allergen is similar in wild-type and A₃AR-deficient mice, and that overexpression of this receptor in Clara cells neither induces mucin production itself, nor enhances mucin production in response to allergen challenge. Collectively, these experiments demonstrate that the A₃AR is neither necessary nor sufficient for mucous cell metaplasia. In contrast to the lack of effect on mucin production, agonist-induced mucin secretion was increased in goblet cells overexpressing the A₃AR, and was absent in A₃AR-deficient mice. Thus, the A₃AR contributes to mucin secretion in allergen-induced metaplasia. Signaling through this receptor may contribute to mucus airway obstruction seen in pulmonary disorders in which adenosine levels are elevated.

Keywords: mucin; mucous cell metaplasia; secretion; adenosine receptors; allergic lung disease

Overproduction and hypersecretion of mucin are prominent features of chronic lung diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (1–3). Obstruction of the airways by mucus is a major contributor to morbidity in patients with these pulmonary conditions. Mucus obstruction is the result of two distinct processes: mucous cell metaplasia, and the increased secretion of mucin (4). Mucous metaplasia and mucin secretion are regulated by distinct extracellular signaling molecules, but detailed information into the concerted regulation of these processes in the diseased airway is lacking. Thus, investigation into basic cellular mechanisms involved in these processes is needed to clarify how coordinate regulation of mucin production and secretion lead to airway obstruction.

Adenosine is a nucleoside that is generated at high levels during situations of cellular stress or injury (5). Consistent with this, adenosine levels are elevated in the airways of individuals with asthma (6), in whom the degree of adenosine elevation correlates with the magnitude of pulmonary inflammation (7). Adenosine has been implicated in chronic lung diseases such as asthma and COPD (8, 9), in part by promoting mast cell degranulation (10), cytokine and chemokine expression (11), and airway hyperresponsiveness (12). Furthermore, a recent study by McNamara and colleagues demonstrates that adenosine within tracheal aspirates from subjects with asthma stimulates the transcription of the gel forming mucin genes MUC5AC and MUC2 in airway epithelial cells (13). These findings have important implications for the ability of endogenously generated adenosine to impact mucin production in the asthmatic airway. Moreover, studies in experimental models of chronic lung disease demonstrate that elevations of adenosine are closely associated with increased mucin production and secretion (14, 15). Collectively, these studies suggest that adenosine produced in the inflamed lung contributes to mucus obstruction in the airways; however, the signaling pathways involved are not yet known.

Adenosine elicits its cellular responses by engaging cell surface receptors (16). There are four such adenosine receptors (A₁AR, A_{2A}AR, A_{2B}AR, and A₃AR), all of which are seven membrane-spanning G protein-coupled receptors. Adenosine receptors are widely distributed in tissues and have unique affinities for adenosine and its analogs. Engagement of adenosine receptors serves to regulate homeostasis in many physiologic systems, and can impact inflammation and tissue injury in many disease states (5, 16, 17). The A₃AR receptor has received attention in asthma due in part to findings that the levels of this receptor are elevated in the airways of patients with asthma (18). In addition, recent studies have shown that levels of the A₃AR are substantially elevated in the lungs of mice in experimental models of Th2 and adenosine-dependent lung injury (15, 19). Cellular localization of the A₃AR in these models revealed that the A₃AR is not expressed in epithelial cells of normal bronchial airways; however, it is upregulated in mucin-producing Clara cells within the airways of diseased mice. The function of the A₃AR in mucin-producing epithelial cells is not known; however, its upregulation suggests that it is involved in aspects of mucous cell biology.

The purpose of the current study is to use genetic approaches to investigate the contribution of A₃AR signaling to mucin production and secretion in a mouse model of allergen-induced pulmonary disease. Results demonstrate that the degree of mucin production in response to allergen is not altered by the genetic removal or the overexpression of the A₃AR in Clara cells. Further, overexpression of the A₃AR in Clara cells did not lead to mucous cell metaplasia, even in the presence of an A₃AR agonist. These experiments demonstrate that the A₃AR is neither necessary nor sufficient for mucous cell metaplasia. In contrast, agonist-induced mucin secretion was increased in Clara cells expressing endogenous or transgenically elevated levels of the A₃AR, while there was a lack of agonist-induced mucin

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secretion in A_3AR -deficient ($A_3AR^{-/-}$) mice. Thus, the A_3AR is involved in mucin secretion in response to allergen-induced metaplasia, and may thus contribute to mucus airway obstruction seen in various pulmonary disorders.

MATERIALS AND METHODS

Mice

A_3AR -deficient ($A_3AR^{-/-}$) mice were generated as previously described (20) and were obtained from Merck Research Laboratories (West Point, PA). Mice were congenic on the C57BL/6J background, and wild-type ($A_3AR^{+/+}$) C57BL/6J mice were purchased from Taconic Laboratories (Germantown, NJ). Genotypes were determined by PCR analysis of genomic DNA obtained from tails at weaning using the following primers: $A_3AR^{+/+}$ (sense: 5'-ATGGAAGCCGACAACACCAC-3', antisense: 5'-CTACAGCAATGGCCAGCAAG-3'), $A_3AR^{-/-}$ (sense: 5'-CTGAG TTGATGGTCTGTA-3', antisense: 5'-GCCTTCTTGACGAGTTCTTC-3'). For the generation of A_3AR -overexpressing mice, a 7.5-kb *Clal* restriction fragment containing the entire A_3AR gene was cloned into the *Clal* site of the plasmid containing a 2.3-kb fragment containing the promoter region of the rat Clara cell secretory protein CC10 (gift from Dr. Jamie Lee, Mayo Clinic, Scottsdale, AZ). This transgenic construct (CC10- A_3AR) was excised from plasmid sequences by *NotI* digestion and injected into C57BL/6J one-cell embryos. Transgenic founder mice were identified from genomic PCR analysis of tail DNA using the following primers: CC10- A_3AR transgene (sense: 5'-TGCTCGAGCTCTAGATATCG-3', antisense: 5'-ATCTTGACTTGACGCTGAC-3'). All mice were maintained in microisolator cages housed under specific pathogen-free conditions. Sentinel mice within the colony surveyed negative for viral antibodies and the presence of known mouse pathogens. All experiments in mice were approved by the University of Texas Health Science Center at Houston Animal Welfare Committee.

Ovalbumin Sensitization and Challenge and IB-MECA Exposures

Six- to eight-week-old $A_3AR^{+/+}$, $A_3AR^{-/-}$, or CC10- A_3AR mice were sensitized by an intraperitoneal injection of 500 μ l ovalbumin(OVA)/AlOH₃ (8 μ g/ml OVA and 8 mg/ml AlOH₃; Sigma-Aldrich, St. Louis, MO) on Days 0, 7, and 14. Mice were challenged by intranasal installation of 30 μ l of either saline or OVA (8 mg/ml) on Days 14, 21, 22, and 23. For IB-MECA exposures, 6-wk-old naïve or OVA-sensitized and -challenged mice were exposed to aerosolized saline or 1 mM IB-MECA for 5 min on Day 24. IB-MECA is a selective A_3AR agonist (21), and the exposure protocol described above has been used to engage A_3AR s in the mouse airway (22).

RNA Isolation and Quantitative RT-PCR Analysis

RNA was isolated from whole lung tissue using Trizol reagent (Invitrogen, Grand Island, NY). RNase-free DNase was used to eliminate genomic DNA contamination (Invitrogen), and RNA quality was assessed by electrophoresis through formaldehyde agarose gels. Transcript levels were quantified using real-time quantitative RT-PCR. Adenosine receptor and β -actin transcripts were analyzed by using Taqman probes or SYBER green on a Smart Cycler (Cepheid, Sunnyvale, CA), with primer sequences and conditions as previously described (19, 23, 24). Data were analyzed using Smart Cycler (Cepheid) analysis software. To generate a standard curve, PCR amplification was performed with template dilutions for each transcript with the assistance of the Smart Cycler program (Cepheid) to determine concentration by the cycle number where it crosses the threshold (C_t). Final data were normalized to β -actin.

In Situ Hybridization

The cDNA clones used to generate riboprobes for the mouse A_3AR were obtained from Marlene Jacobson (Merck Research Laboratories). Plasmids were linearized and either T₃ or T₇ RNA polymerases were used to generate antisense or sense riboprobes labeled with digoxigenin. Formalin-fixed, paraffin-embedded lungs were sectioned (5 μ m) and collected on Superfrost/plus positively charged microscope slides (Fischer Scientific, Houston, TX). The sections were deparaffinized

and rehydrated according to a standard protocol and fixed in 4% paraformaldehyde for 15 min at room temperature. Sections were treated with 0.1% Triton X-100 for 10 min at room temperature, then washed in 200 mM HCl for 5 min. Sections were digested with 5–10 μ g/ml of proteinase K in 20 μ M Tris and 2 mM CaCl₂ at 37°C for 20 min. Sections were postfixed in 4% paraformaldehyde for 5 min at room temperature, washed in 0.5% acetic anhydride in Tris (pH 8.0) for 10 min, and hybridized overnight at 60°C in 100 μ l of hybridization buffer (2 $\times$ standard saline citrate, 50% formamide, 10% dextran sulfate, and 0.02% sodium dodecyl sulfate) and 1.5 μ l of antisense riboprobe per section. Hybridization using sense riboprobes was performed under identical conditions. Sections were washed three times in 50% formamide in saline citrate at 60°C for 20 min each, and then washed in saline citrate two times for 15 min. Detection was performed using anti-digoxigenin alkaline phosphatase (Roche, Indianapolis, IN) and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) as substrate (Invitrogen). Sections were counterstained in nuclear red, coverslipped, and viewed and photographed using an Olympus BX60 microscope equipped with a SPOT digital camera (Diagnostics Instrument, Sterling Heights, MI).

Membrane Preparations and Receptor Binding Assays

Crude membrane preparations were made from whole lungs collected from 6-wk-old mice. Frozen lung tissue was minced with scissors in an ice-cold buffer containing 0.32 M sucrose and 25 mM Tris (pH 7.4 at 37°C), and homogenized using a polytron (PT2100, Brinkmann) with two 30-s bursts at a low setting. The homogenate was centrifuged at 1,000 $\times g$ for 10 min at 4°C (Sorvall SS-34, New Town, CT). The filtered supernatant was centrifuged at 40,000 $\times g$ for 20 min at 4°C. The pellet was resuspended in ice-cold 25 mM Tris-HCl buffer (pH 7.4) and centrifuged at 40,000 $\times g$ for 20 min at 4°C. The final pellet was resuspended in 200 μ l 25 mM Tris-HCl (pH 7.4), and membrane protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL). Next, 20 μ g of the membrane suspension was used for binding assays conducted in 96-well plates. For saturation binding, a range of [¹²⁵I] AB-MECA was added to each well, and 25 mM Tris was added to a final volume of 100 μ l. The nonselective AR agonist NECA (100 μ M) was used to determine nonspecific binding. After incubation for 1 h at 37°C, cell membranes were retained and washed on a 96-well filter plate. Radioactivity was counted using a γ counter.

Immunohistochemical Analysis

Paraffin-embedded lungs were sectioned (5 μ m) and collected on Superfrost/plus positively charged microscope slides. Deparaffinized sections were rehydrated in a series of graded alcohols to water. Endogenous peroxidase activity was blocked by incubation in 0.5% H₂O₂ in methanol for 20 min. Sections were then incubated for 1 h at room temperature with a 1:20,000 dilution of rabbit anti-mouse CC10 kD protein (gift from Franco DeMayo, Baylor College of Medicine, Houston, TX) followed by development with a rabbit IgG Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA) and counterstaining with methyl green.

Mucin and Secretion Index

Sections (5 μ m) were stained with PAFS reagent as described (25). Briefly, sections were placed in 1% periodic acid for 10 min at room temperature. Sections were then washed in water and placed in fluorescent Schiff reagent (0.5% acriflavin, 1% potassium metabisulphite, and 0.1 M HCl) for 20 min at room temperature. Sections were then washed in tap water and placed in acid alcohol (0.1 M HCl in 70% EtOH) for two changes of 5 min each. Sections were then dehydrated and coverslipped. A mucin index was calculated by dividing the area of PAFS staining by the total area of the airway epithelium as described (14). In this analysis, multiple airways in the lung, with and without mucin, were analyzed to provide an overall assessment of mucin production in the lungs. A mucin secretion index was determined by examining only mucin containing airway epithelium at $\times 40$ magnification. The area of PAFS staining was divided by the total area of the airway epithelium in regions that contained mucin. This index was used to monitor the amount of mucin within individual cells, with decreases in the degree of PAFS staining being indicative of mucin secretion into the airways (25).

RESULTS

The A₃AR Is Not Required for Mucous Metaplasia

Elevations in adenosine, as well as increased expression of the A₃AR, are seen in the airways of animal models exhibiting mucous metaplasia (14, 15, 19). To examine the necessity of the A₃AR in mucin production, changes in PAFS staining were assessed in A₃AR wild-type (A₃AR^{+/+}) and A₃AR^{-/-} mice subjected to OVA sensitization and challenge. A₃AR^{+/+} and A₃AR^{-/-} mice sensitized with OVA and challenged with saline had no mucin production in their airways (Figures 1A and 1C). In contrast, OVA-sensitized and -challenged A₃AR^{+/+} and A₃AR^{-/-} mice showed significant mucin production in their airways (Figures 1B and 1D, *orange staining*). Quantification of the PAFS staining revealed no significant differences in the amount of mucin produced in the airways of A₃AR^{+/+} and A₃AR^{-/-} mice (Figure 1E). Similarly, there was no difference in degree of expression of the major mucin, Muc5ac (Figure 1F), and the mucous metaplasia

marker calcium-activated chloride channel 3 (Clca3) (Figure 1G). These results demonstrate that the A₃AR is not necessary for the development of the mucous phenotype after allergen challenge in the mouse.

Generation and Characterization of Mice Overexpressing the A₃AR in the Airways

To determine if A₃AR signaling is sufficient to induce mucous metaplasia and mucin production, a line of transgenic mice overexpressing the A₃AR in the airways was generated. The rat Clara cell 10-kD protein (CC10) promoter was used to target expression of an A₃AR minigene to the airways (Figure 2A). A transgenic line containing nine copies of the CC10-A₃AR transgene (data not shown) was identified (Figure 2B) and characterized for increased expression of the A₃AR in Clara cells. CC10-A₃AR transcript levels were elevated 200-fold in lungs of naïve transgenic mice (Figure 2C). Furthermore, A₃AR overexpression from this transgene was predominantly lung specific,

Figure 1. The A₃AR is not required for mucin production after allergen challenge. Mucin production was determined by PAFS staining of lung sections. (A) Airway from an A₃AR^{+/+} mouse sensitized with OVA and challenged with saline. (B) Airway from an A₃AR^{+/+} mouse sensitized and challenged with OVA. (C) Airway from an A₃AR^{-/-} mouse sensitized with OVA and challenged with saline. (D) Airway from an A₃AR^{-/-} mouse sensitized and challenged with OVA. Arrows denote mucin-producing cells; scale bars = 100 μm. (E) Mucin production was quantified by measuring PAFS staining as described in MATERIALS AND METHODS. Values are presented as mean mucin index score ± SEM (*n* = 4). Whole lung RNA extracts were analyzed using quantitative RT-PCR assays for Muc5ac (F) and Clca3 (G). o/s, OVA sensitization and saline challenge; o/o, OVA sensitization and challenge. Values are presented as transcript level (% β-actin) ± SEM; *n* = 4 (**P* < 0.05 A₃AR^{+/+} o/o and A₃AR^{-/-} o/o versus A₃AR^{+/+} o/s using Student's *t* test).

Figure 2. Generation, genotyping, and characterization of CC10-A₃AR transgenic mice. (A) The transgene was generated by the addition of the A₃AR gene into a plasmid that contained the rat CC10 promoter and a β-globin intron and poly A sequences. The transgene was excised from the plasmid by NotI digestion. (B) Genotypes were determined by PCR analysis using forward and reverse primers specific for the CC10-A₃AR construct. Positive control is the plasmid used to create the transgenic mice. (C) Isolated RNA from lung, liver, heart, and kidney from A₃AR^{+/+} and CC10-A₃AR mice were analyzed using a Taqman-based quantitative RT-PCR assay for the A₃AR transcript. Values are presented as transcript level (% β-actin) ± SEM; n = 5. (D) Binding assays were performed on whole lung crude membrane preparations from A₃AR^{+/+} and CC10-A₃AR mice. Saturation binding was determined from the addition of [¹²⁵I] AB-MECA (0.1–4 nM). Specific binding was determined by the addition of NECA (not shown). Values are presented as mean bound ± SEM; n = 3 in triplicate.

with only slight expression seen in other tissues (Figure 2C). Increased expression was further verified by conducting A₃AR saturation binding assays on cell membranes isolated from the lungs of CC10-A₃AR transgenic mice (Figure 2D). Lungs from naïve A₃AR^{+/+} mice exhibited no specific binding even at the highest concentration of the A₃AR-selective analog [¹²⁵I] AB-MECA (Figure 2D). In contrast, lungs from naïve CC10-A₃AR transgenic mice demonstrated a substantial increase in A₃AR binding with a saturation binding curve that gave a K_d of 1.14 nM. Scatchard plot analysis revealed the B_{max} to be 0.133 fmol/mg. These findings confirm that A₃AR binding is elevated in the lungs of CC10-A₃AR transgenic mice.

Localization of A₃AR in the airways of transgenic mice was analyzed using *in situ* hybridization. Whereas bronchial epithelium from naïve A₃AR^{+/+} mice showed no A₃AR signal (Figure 3A), the bronchial epithelium of naïve CC10-A₃AR transgenic mice exhibited strong signal for the A₃AR (Figure 3B). The majority of the cells in the airways of A₃AR^{+/+} and CC10-A₃AR transgenic mice were shown to be Clara cells by immunohistochemical analysis with a CC10 antibody (Figures 3E and 3F). These data demonstrate that the A₃AR is elevated in Clara cells of CC10-A₃AR transgenic mice.

Effects of A₃AR Overexpression on Mucin Production

To assess the impact of increased A₃AR expression on mucin production, lungs were isolated from CC10-A₃AR transgenic

mice and stained histochemically for mucin using PAFS. PAFS-positive staining was not detected in the airways of naïve A₃AR^{+/+} (Figure 4A) or CC10-A₃AR (Figure 4C) mice. These findings indicate that the increased expression of the A₃AR in Clara cells is not sufficient for the induction of mucin production. Since endogenous adenosine levels are low in uninjured lungs (14), the absence of mucin in the lungs of naïve CC10-A₃AR mice could likely reflect the absence of sufficient adenosine levels to engage these receptors. To address this, mice were exposed to aerosolized IB-MECA, a selective A₃AR agonist (21). However, PAFS-positive staining was not detected in the airways of naïve A₃AR^{+/+} (Figure 4B) or CC10-A₃AR (Figure 4D) mice exposed to IB-MECA. Similarly, there was not an increase in mucus scores or the expression of Muc5ac and Clca3 in these mice (Figures 4E–4G). Together, these results indicate that the overexpression of the A₃AR is not sufficient to induce Clara cells to produce mucin, even in the presence of an agonist.

Effects of A₃AR Overexpression on Mucin Secretion

To examine the impact of A₃AR signaling on mucin secretion, mice were sensitized and challenged with OVA and basal to apical distributions of mucin staining were monitored. Airway mucous cells from OVA-sensitized and -challenged A₃AR^{+/+} mice appeared oval and laden with PAFS-positive granules (Figure 5A) distributed evenly through the cytoplasm. Upon treatment with IB-MECA, total PAFS staining was reduced and

Figure 3. Localization of the A₃AR in CC10-A₃AR transgene in unchallenged mice. Lung sections from CC10-A₃AR transgenic (B, D, and F) and A₃AR^{+/+} (A, C, and E) mice were hybridized with A₃AR antisense (A and B) and sense (C, and D) digoxigenin-labeled RNA probes. A₃AR^{+/+} mice exhibit no specific A₃AR signal (purple) in bronchial epithelial cells (A) that are shown to be Clara cells by positive immunohistochemical staining for CC10 (αCC10) in a neighboring section (E). By contrast, A₃AR transcript was abundant in the airway epithelial cells from CC10-A₃AR mice (B), which also show positive staining for the CC10 protein (F). Scale bar in figures = 100 μm; scale bar in insets = 30 μm.

the distribution of residual mucin was shifted to a more apical distribution in OVA-sensitized and -challenged A₃AR^{+/+} mice (Figure 5B), implying that IB-MECA induces secretion. Similarly, compared with OVA-sensitized and -challenged A₃AR^{+/+} mice, OVA-sensitized and -challenged CC10-A₃AR transgenic mice showed reduced PAFS staining. This was characterized by a loss of perinuclear mucin localization, with the remainder of the mucin showing localization at the epithelial apices (Figure 5C). Likewise, secretion was enhanced further in A₃AR-CC10 transgenic mice after IB-MECA exposure (Figure 5D). These findings suggest there is enhanced steady state and evoked mucin secretion in mice overexpressing the A₃AR. Furthermore, mucous cells in the airways of OVA-sensitized and -challenged A₃AR^{-/-} mice showed no evidence of altered cellular mucin morphology, even after IB-MECA exposure (Figures 5E and 5F), thus confirming that selective A₃AR stimulation results in secretion of mucin from airway mucous cells. Morphometric analysis of these histologic observations was conducted to establish a quantitative measure of mucin secretion. This analysis confirmed that there was an acute reduction in intracellular mucin in A₃AR^{+/+} mice after IB-MECA treatment (Figure 6), indicating evoked secretion. Furthermore, mucous cells in the airways of CC10-A₃AR transgenic mice had lower levels of

steady-state mucin than A₃AR^{+/+} mice, and activation with IB-MECA enhanced secretion (Figure 6). Finally, A₃AR^{-/-} mice exhibited a higher level of intracellular mucin than A₃AR^{+/+} mice, and showed no secretion after IB-MECA exposure. Together, these data suggest that engagement of the A₃AR promotes mucin secretion *in vivo*.

To confirm that the above alterations in mucous cell morphology were not due to alterations in mucin production, the expression of genes associated with the mucous phenotype were examined. Analysis of RNA extracts from whole lungs revealed that there were similar increases in Muc5ac (Figure 7A) and Clca3 (Figure 7B) in the lungs of A₃AR^{+/+}, CC10-A₃AR transgenic, and A₃AR^{-/-} mice sensitized and challenged with OVA. These findings indicate that the increased mucin secretion seen was not related to changes in mucin expression phenotype. Similarly, there was not a significant difference in the degree of inflammatory cell infiltration (data not shown), or cytokine (Figure 8A) and chemokine (Figure 8B) levels in the lungs of A₃AR^{+/+}, CC10-A₃AR transgenic, or A₃AR^{-/-} mice sensitized and challenged with OVA. These data indicate that altered inflammation and mediator production is not the cause of altered IB-MECA-stimulated mucin secretion in these genetic models.

Figure 4. Mucin production in the airways of $CC10-A_3AR$ transgenic mice. Mucin production was determined by PAFS staining lung sections. (A) Airway from an $A_3AR^{+/+}$ mouse challenged with aerosolized saline. (B) Airway from an $A_3AR^{+/+}$ mouse challenged with aerosolized IB-MECA. (C) Airway from a $CC10-A_3AR$ mouse challenged with aerosolized saline. (D) Airway from a $CC10-A_3AR$ mouse challenged with aerosolized IB-MECA. Scale bars = 100 μ m. (E) Mucin production was quantified by measuring PAFS staining as described in MATERIALS AND METHODS. Values are presented as mean mucin index score $\pm$ SEM; $n = 3$. Whole lung RNA extracts were analyzed using quantitative-RT-PCR assays for *Muc5ac* (F) and *Clca3* (G). S, saline; IB, IB-MECA. Values are presented as transcript level (% β -actin) $\pm$ SEM; $n = 3$.

DISCUSSION

The cellular mechanisms involved in increased airway mucin production and secretion are not well understood. This study was designed to use genetic approaches to examine the involvement of the A_3AR in these processes using a standard mouse model of allergen-induced lung disease. Our findings demonstrate that the A_3AR is not involved in the production of mucin by airway mucous cells of allergen-challenged mice, but does contribute to mucin secretion in the airways. Thus, adenosine signaling through the A_3AR may contribute to airway mucus obstruction by contributing to the hypersecretion of mucin.

Adenosine levels are controlled in tissues and cells by the enzyme adenosine deaminase (ADA) (26). Mice that lack this enzyme develop severe pulmonary inflammation and airway remodeling in association with elevations in lung adenosine content. Mucin overproduction and hypersecretion are features of this model, and the use of ADA replacement therapy to lower adenosine levels can reverse the degree of mucin production seen (14). Similar associations between adenosine elevations and mucin overproduction are seen in the airways of mice that overexpress the Th2 cytokine IL-13 (15). In addition, adenosine levels are elevated in the lungs of individuals with asthma (6), where mucin overproduction is prominent (1). Hence, there is a close relationship between elevations in lung adenosine concentrations and increased mucin production. In pursuit of the mechanisms by which adenosine might mediate mucin pro-

duction, we turned our attention to the A_3AR for several reasons. The A_3AR is upregulated in biopsies taken from the airways of individuals with asthma (18), and levels of this receptor are elevated in the lungs of ADA-deficient and IL-13-overexpressing mice (15, 19). Localization of A_3AR expression in the airways demonstrates that it is not found in non-mucin-producing airway epithelial cells, whereas it is upregulated in airway epithelial cells that stain for mucus (15, 19). Lastly, genetic removal of the A_3AR from ADA-deficient mice was associated with decreased mucin production (19). Collectively, these data led to the hypothesis that the A_3AR mediates adenosine-dependent mucin production in the airways. Results from the current study suggest that this hypothesis is null in mice where mucin production is stimulated in response to allergen exposure. $A_3AR^{+/+}$ and $A_3AR^{-/-}$ mice sensitized and challenged with OVA exhibit the same degree of airway mucin production and mucin-associated gene expression in the lungs. Furthermore, overexpression of the A_3AR in mucin-producing cells was neither sufficient for inducing mucin production in the naïve airway nor sufficient for increasing levels of mucin production in OVA-challenged mice, suggesting that this receptor is likely not involved in the development of mucous cell metaplasia in allergen-challenged mice. Thus, it appears that the A_3AR is neither necessary nor sufficient for the production of mucin in response to allergen.

What mechanisms then, if any, might regulate adenosine's effects on mucin production? It is likely that adenosine may

Figure 5. Morphologic changes in mucin-producing cells after IB-MECA exposure. Mucin distribution was determined by PAFS staining of lung sections. (A) Airway of an OVA-sensitized and -challenged A₃AR^{+/+} mouse exposed to saline. (B) Airway of an OVA-sensitized and -challenged A₃AR^{+/+} mouse exposed to IB-MECA. (C) Airway of an OVA-sensitized and -challenged CC10-A₃AR mouse exposed to saline. (D) Airway of an OVA-sensitized and -challenged CC10-A₃AR mouse exposed to IB-MECA. (E) Airway of an OVA-sensitized and -challenged A₃AR^{-/-} mouse exposed to saline. (F) Airway of an OVA-sensitized and -challenged A₃AR^{-/-} mouse exposed to IB-MECA. Images are representative of those found in four animals per each condition. Arrows depict basolateral to apical direction of the airway epithelium. Scale bar in figures = 30 μ m. Enlarged insets were taken at $\times 100$ magnification.

influence mucin production through interaction with adenosine receptors other than the A₃AR. A recent study has shown that adenosine within tracheal aspirates of individuals with asthma can lead to the upregulation of mucin gene expression in human airway epithelial cells *in vitro* (13). In that study, pharmacologic agents were used to implicate the involvement of the A₁AR in stimulating mucin gene expression through a mechanism that involved activation of the calcium²⁺-activated chloride channel CLCA1. Thus, the A₁AR may govern aspects of adenosine-mediated mucus production through engagement of the A₁AR. However, recent studies demonstrating enhanced mucin production in the airways of ADA-deficient mice lacking the A₁AR (24) would argue against such a mechanism. The A_{2B}AR is a low-affinity receptor that is also expressed in the airway epithelium of mice overproducing mucin (15), making it a possible candidate for mediating the direct effects of adenosine on mucin production. It is also possible that adenosine may contribute to increased mucin production indirectly by promoting aspects of pulmonary inflammation. Consistent with this, adenosine has been shown to promote mast cell degranulation (10, 27) and airway eosinophilia (14, 19), both of which can impinge on mucin production and obstruction in the airways (28, 29). Moreover,

adenosine can stimulate production of IL-13 (15, 30), a potent cytokine known to directly influence the production of mucin in the airways (31, 32). The decreased mucin production seen in ADA/A₃AR double knockout mice (19) likely results from attenuation of one of these aforementioned pathways. Individual adenosine receptors will have to be examined in the context of specific disease and inflammatory conditions to fully appreciate their relative involvement in mucin production. Doing so will help to clarify the involvement of this signaling nucleoside in the production of mucin.

In contrast to effects on mucin production, results from the current study demonstrate that the A₃AR plays an important role in the secretion of mucus from goblet cells in the airways of allergen-challenged mice. Exposure to the A₃AR agonist IB-MECA induced mucin secretion as determined by decreased intracellular mucin content, and apical localization of remaining mucin containing vesicles within goblet cells. This effect was increased after the overexpression of the A₃AR in Clara cells, and IB-MECA-stimulated secretion was not detected in mucin-producing goblet cells in the airways of allergen-challenged A₃AR^{-/-} mice. The levels of Muc5Ac and Clca3, two major mucous metaplasia-associated genes, did not change amongst

Figure 6. Mucin secretion after IB-MECA exposure. Mucin secretion was quantified by counting 10 random fields per slide that contained PAFS-positive airway epithelial cells. Secretion index was determined by measuring total area of PAFS staining divided by the total area of the airway epithelium in regions that contained mucin. Values are presented as mean secretion index score $\pm$ SEM; $n = 4$ (* $P < 0.05$ IB-MECA versus saline, # $P < 0.05$ $CC10-A_3AR$ or $A_3AR^{-/-}$ versus $A_3AR^{+/+}$ using a Student's t test).

groups, indicating that mucous metaplasia was not affected whereas secretion was. Thus, upregulation of the A_3AR on mucin-producing goblet cells (15, 19) may represent a mechanism for increasing mucin secretion in chronic lung diseases in which adenosine levels are elevated, such as asthma (6).

Interestingly, there was increased mucin secretion in allergen-challenged mice overexpressing the A_3AR , even in the absence of agonist administration (Figures 5 and 6), suggesting that overexpression of the A_3AR led to increased sensitivity to endogenous adenosine levels. The excessive release of mucin in response to endogenous adenosine levels may account for the relatively modest additional release seen after agonist stimulation. Similarly, the observation that there was persistent mucus present even after agonist stimulation in the lungs of wild-type mice suggests that there may be a pool of mucus that is not accessible to A_3AR stimulation. It is likely that there are a number of secretagogues that work in concert in the inflamed lung to propagate mucus obstruction. For example, ATP and UTP are potent stimulators of mucus secretion (25, 33). Direct comparisons between adenosine, ATP, and UTP *in vivo* would help to clarify this issue.

Consistent with our findings in mice *in vivo*, studies in the canine trachea have shown that adenosine can have a potent effect on mucin secretion (34). However, other studies using isolated epithelial cells in culture have shown that adenosine has little effect on mucin secretion (33, 35). Discrepancies among these findings may stem from differences in the origin of the cell types examined and the environment in which they were surveyed, since A_3AR expression depends on inflammatory signaling (19). To the best of our knowledge, the current study is the first to demonstrate the ability of an adenosine agonist to promote airway mucus secretion *in vivo* under allergic conditions. Future studies examining the ability of adenosine and A_3AR activation to promote mucin secretion in additional *in vivo* models will help to clarify how universal this pathway is in the secretion of mucin.

The mechanism(s) by which A_3AR engagement on goblet cells leads to increased mucin secretion is not known. Most evidence suggests that the A_3AR is coupled to the inhibitory G protein $G_{\alpha i}$, which downregulates cAMP levels by inactivating adenylyl cyclases (16). However, there is evidence to suggest

Figure 7. Muc5ac and Clca3 expression in the lungs of mice with various amounts of A_3AR expression. Whole lung RNA extracts were analyzed using quantitative RT-PCR assays for Muc5ac (A) and Clca3 (B). Values are presented as mean transcript level (% β -actin) $\pm$ SEM; $n = 3$ for each condition (* $P < 0.05$ o/o + IB-MECA and o/o + saline versus o/s + saline using Student's t test).

that activation of the A_3AR plays important roles in aspects of cellular secretion, which is likely mediated by the ability of this G protein-coupled receptor to access effector pathways that promote this process. For example, engagement of the A_3AR on mouse mast cells can lead to degranulation through pathways that involve the $\beta\gamma$ subunits of the receptor, activation of PI3 kinase, and subsequently elevation in intracellular Ca^{2+} levels (22, 36). The mobilization of intracellular Ca^{2+} is critical for regulated secretion of many substances (37) including mucin from airway mucous cells (38, 39). Therefore, engagement of the A_3AR may contribute to increased mucin secretion by leading to increases in Ca^{2+} levels within mucin-producing Clara cells. Activation of protein kinase C (PKC) isoforms is another proposed mechanism for regulated mucin secretion (39). PKC is known to interact and activate myristoylated-alanine-rich C-kinase substrate (MARCKS) (40), which plays an important role in the regulated secretion of mucin in the airways (41). A_3AR engagement can activate PKC (42). Thus, A_3AR activation of PKC may activate MARCKS and contribute to mucin secretion. Defining the involvement of downstream A_3AR signaling pathways in mucin secretion could provide novel targets for the development of therapies to circumvent mucus obstruction in the lung.

There is substantial evidence that inflammatory mediators such as cytokines can lead to mucin overproduction in the airways of experimental models of asthma (31, 32). Once mucin is produced, subsequent mucin secretion into the airways can lead to fatal

Figure 8. Cytokine and chemokine production in the lungs of mice with various amounts of A₃AR expression. Mice were sensitized with OVA and then challenged with saline (–) or OVA. In addition, mice were exposed to aerosolized saline (–) or IB-MECA before harvesting whole lungs for RNA extraction. (A) Cytokine and (B) chemokine production was measured in whole lung extracts using quantitative RT-PCR. Values are presented as mean transcript level (% β-actin) ± SEM; n = 3 for each condition (*P < 0.05 o/o + IB-MECA and o/o + saline versus o/s + saline using Student's t test).

airway obstruction (1). Adenosine levels are increased in the lung as result of inflammation and damage (5). Our current findings suggest that such elevations might access A₃AR-dependent pathways that selectively influence mucin secretion without affecting mucin production. Therefore, the upregulation of the A₃AR expression in the bronchial airways during inflammatory metaplasia (15, 19) may provide a mechanism for promoting the secretion of mucin in injured environments where adenosine levels are elevated. Interestingly, ATP, a metabolic precursor of adenosine, is rapidly released from damaged airway epithelial cells (43), and this adenine nucleotide can stimulate mucin secretion *in vitro* (33, 35, 44) and *in vivo* (25), but not mucin production (33), through engagement of P2 purinergic receptors. Thus, the concerted upregulation of ATP and adenosine levels in the lung may represent an important mechanism of regulated mucin hypersecretion that may contribute to mucus occlusion of the airways.

Conflict of Interest Statement: H.W.J.Y., C.-X.S., C.M.E., and B.F.D. do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.R.B. is a paid consultant with CV Therapeutics for the investigations of adenosine receptor analogs and lung disease.

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