ERJ Express. Published on October 24, 2007 as doi: 10.1183/09031936.00003707

Elevated expression of adenosine A_1 receptor in bronchial biopsies from asthmatic subjects

R.A. Brown[*], G.W. Clarke[#], C.L. Ledbetter[#], M.J. Hurle[¶], J.C. Denyer[¶], D.E. Simcock[#], J.E. Coote[¶], T.J. Savage[¶], R.D. Murdoch[¶], C.P. Page[*], D. Spina[*], B.J. O'Connor[#].

[*]King's College London, Sackler Institute of Pulmonary Pharmacology, Division of Biomedical and Health Sciences, London, United Kingdom.

[#]King's College London, Department of Asthma and Allergy, Division of Medicine, London, United Kingdom.

[¶]GlaxoSmithKline, Stevenage, United Kingdom.

Corresponding Author:

Clive P. Page, Ph.D

King's College London

Sackler Institute of Pulmonary Pharmacology

5th floor, Hodgkin Building, Guy's Campus,

London, U.K., SE1 1UL

Tel: +44 (0)207 8486096

Fax: +44 (0)207 8486097

Email: clive.page@kcl.ac.uk

Funded by a grant from GlaxoSmithKline, Stevenage, United Kingdom.

Word count: 4741

ABSTRACT

Asthmatics, unlike healthy subjects, bronchoconstrict in response to inhaled adenosine and extracellular adenosine concentrations are elevated in bronchoalveolar lavage fluid and exhaled breath condensate of asthmatic subjects. However, little is understood about the location and expression of adenosine receptors in asthmatic airways. Our aim was to first investigate the distribution of adenosine A_1 receptors in bronchial biopsies from mild asthmatic steroid-naïve subjects and then compare the degree of expression with healthy subjects.

Biopsy sections were immunostained with an adenosine A_1 receptor antibody, the selectivity of which was validated in specific experiments. Image analysis was then performed to determine differences in immunostaining intensity.

Immunostaining of biopsies from the asthmatic subjects revealed strong expression of the A_1 receptor located predominantly to the bronchial epithelium and bronchial smooth muscle. In comparison, very weak immunostaining was observed in biopsies obtained from healthy subjects. Image analysis revealed that the intensity of positive staining on the asthmatic bronchial epithelium and smooth muscle regions was significantly greater than that observed with the healthy epithelium and smooth muscle ($P < 0.01$).

In conclusion, the sensitivity of asthmatics to inhaled adenosine coupled with increased adenosine A_1 receptor expression implicates a role for these receptors in the pathophysiology of this disease.

Key Words-Adenosine, asthma, adenosine A_1 receptor, adenosine A_1 receptor expression

INTRODUCTION

Adenosine is a purine nucleoside that is expressed in all cells of the body and involved in a wide range of physiological processes. The effects of adenosine are mediated predominantly through specific cell surface receptors of which four subtypes (A_1, A_{2A}, A_{2B} and A_3) have been described. It is now well recognised that extracellular levels of adenosine markedly increase under metabolically stressful conditions, such as hypoxia and inflammation, and whilst an acutely elevated level of extracellular adenosine is considered to mediate anti-inflammatory and protective effects, a chronic accumulation has been associated with pathological consequences [1].

In asthmatic subjects, it has been demonstrated that adenosine levels in bronchoalveolar lavage fluid and exhaled breath condensate are significantly higher than those present in healthy subjects [2, 3], and current evidence strongly suggests that it may contribute to the pathophysiology of asthma. For example, it has been recognised for many years that inhalation of adenosine 5'-monophophate (AMP) (5′-nucleotidase in the lung rapidly hydrolyses AMP to adenosine) in asthmatic but not healthy subjects results in dose-related bronchoconstriction [4], which is considered to be mediated predominantly, but not exclusively by, mast cell degranulation *via* A_{2B} receptor activation (reviewed in 5). Furthermore, inhalation of AMP has been shown to increase airway eosinophilia [6].

A role for adenosine in asthma is further supported by the observation that plasma adenosine levels rapidly increase following allergen challenge in asthmatic subjects [7], suggesting a possible involvement of endogenous adenosine in the early-phase

airway response to allergen. This possibility is supported in allergic rabbits where allergen-induced bronchoconstriction was blocked through the use of an anti-sense oligonucleotide directed against the A_1 receptor [8] and very recently with a selective adenosine A_1 receptor antagonist [9]. Furthermore, it has been demonstrated that the concentrations of adenosine are increased in both plasma and exhaled breath condensate during exercise-induced bronchoconstriction in subjects with asthma [10, 11], and finally, it has also been shown that isolated human asthmatic bronchial tissue contracts in response to adenosine *via* an A_1 receptor dependent mechanism [12].

In order to further investigate the role of endogenous adenosine in asthma, it is crucial that we extend our knowledge concerning the distribution and specific functions of adenosine receptors in asthmatic airways. To date, binding studies in healthy peripheral lung tissue have suggested that A_2 receptor subtypes are more abundant than the A_1 and A_3 receptor subtypes [13], and expression of the A_3 receptor was observed to be upregulated in asthmatic lung tissue, where it was located predominantly to eosinophils [14]. Therefore, our aim was to extend these observations. Although we ultimately intend to profile the expression of all four adenosine receptor subtypes and identify the subtype specific cellular functions, we hypothesized that characterising the distribution and expression of the A_1 receptor would particularly further our understanding of the role of adenosine in asthma given the observations detailed above. Thus, the distribution of the adenosine A_1 receptor in bronchial biopsies from asthmatic subjects was investigated and then compared to the distribution and level of expression with biopsies obtained from healthy subjects in order to provide further insight into the role of adenosine in asthma.

METHODS

Further details of all methods and procedures can be found in the online depository.

Subjects

All of the asthmatic volunteers gave a clinical history consistent with intermittent, reversible airflow obstruction, and atopy to common aeroallergens. Thus, all of the asthmatic subjects (non-smoking) were characterised on the basis of a previous history of wheeze, a forced expiratory volume in 1 second (FEV_1) greater than 70% predicted, atopy as defined by a positive cutaneous response to intradermal allergen, and airways hyperresponsiveness to both methacholine and AMP. In addition, all asthmatic subjects demonstrated an increase in baseline FEV_1 of at least 15-20% in response to inhaled salbutamol (data not shown). Sixteen mild asthmatic subjects were screened for this study and twelve went on to complete bronchial provocation challenge with AMP and bronchoscopy (four subjects withdrew their consent to undergo bronchoscopy). Each subject had infrequent symptoms, controlled with occasional inhaled short-acting $ß_2$-agonists alone. No subject was taking any regular anti-asthma therapy and none had taken inhaled corticosteroids for at least 3 months prior to entry. None of the subjects had an exacerbation of asthma or respiratory infection in the preceding 6 weeks and all subjects were non-smokers. Patient demographics are summarised in Table I. For comparison, biopsies from eight healthy age-matched non-smoking subjects obtained in a separate but recent previous study under the same conditions were also analysed. The demographics from these subjects are described in Table 1. Written informed consent was obtained from each subject and the study was approved by the Ethics Committee of King's College Hospital.

Study design

Procedures at Visit 1 for all subjects included medical history and physical examination, an asthma characterisation questionaire, intradermal skin prick tests, spirometry and methacholine challenge to determine provocative concentration $(PC)_{20}$ values. After approximately 1 week, asthmatic subjects returned for Visit 2 where they underwent AMP challenge to determine the PC_{20} to AMP. All subjects refrained from using rescue medication and caffeinated beverages for at least 8 hours before each visit. After a period of 3-4 weeks, all subjects returned for Visit 3 at which bronchoscopy was performed.

Immunohistochemistry

Using the methods described below to validate the adenosine receptor subtype specificity of an antibody, we found that the specificity of commercially available antibodies against all adenosine receptor subtypes was unsatisfactory, hence an antibody against the adenosine A_1 receptor was created specifically for the present study (the costs involved in obtaining a custom-made antibody was also a contributing factor when deciding not to concurrently profile expression of the other adenosine receptor subtypes). Thus, the custom-made affinity purified A_1 receptor polyclonal antibody was raised in the rabbit against a synthetic peptide corresponding to the amino acid sequence 309-326 (CQPAPPIDEDLPEERPDD) of the COOH-terminus of the human A_1 receptor (Cambridge Research Biochemicals, Cleveland, U.K.).

Brain cortex and cardiac tissue sections from 3 subjects were co-analysed as a positive control, as it is well established that these tissues highly express A_1 receptors.

Cadaveric brain cortex and cardiac tissue samples were obtained from the Institute of Psychiatry Brain Bank and Peterborough Tissue Bank respectively, following GlaxoSmithKline and United Kingdom guidelines for the acquisition and use of human tissues, including ethical approval and the use of appropriate consent forms.

Frozen biopsies of brain cortex, cardiac tissue and bronchial tissue were sectioned at 6 μm. Sections were placed on Vectabond™ coated slides and the brain cortex and cardiac tissue sections fixed in ice-cold 4% w/v paraformaldehyde for 5 min (bronchial biopsies were fixed with 4% w/v paraformaldehyde prior to cryopreservation). Sections were then immunostained for the A_1 receptor using an Optimax automatic immunostaining machine (Biogenex, San Ramon, CA, USA). Positive staining was detected with diaminobenzidine in addition to counterstaining with Mayer's haematoxylin.

Image analysis

Fully automated densitometry of A_1 receptor expression was developed with the Zeisss Vision KS400 system (Carl Zeiss, Gottingen, Germany). Stain intensity is described in arbitrary units (A.U.). All image analysis and measurements were performed blind by one observer.

Antibody validation with adenosine receptor transfected cells

Further antibody validation experiments were performed through flow cytometric analysis of the A_1 receptor antibody binding to each adenosine receptor subtype

expressed on CHO-K1 cells (which lack any known subtype of adenosine receptor) transfected with the human recombinant A_1, A_{2A}, A_{2B} or A_3 receptor. Comparative expression levels of the adenosine receptor subtype in each cell line were first investigated using real-time RT-PCR. Data are expressed as gene copy number per 50 ng cDNA. For flow cytometric analysis, cell lines were analyzed following antibody labelling by appropriate gating for immunofluorescence using a FACS Calibur flow cytometer (Beckton Dickinson, Oxford, U.K.) after excitation at 488 nm. At least 3,000 events were collected and mean fluorescence intensity values recorded.

Statistics

Data were analysed by unpaired Student's t-test, and unless stated otherwise, are expressed as mean ± SEM.

RESULTS

Subjects

Patient demographics are described in Table I. All asthmatic subjects who completed the study were hyperresponsive to AMP and methacholine (Table I). PC_{20} values to methacholine in the eight non-atopic healthy subjects were all greater than 16 mg ml^{-1}. All asthmatic subjects exhibited a positive skin response to at least one allergen, whilst none of the healthy subjects responded to any of the allergens tested.

Immunohistochemistry

Positive immunostaining appeared brown against a blue background resulting from Mayer's haematoxylin counterstain. Immunostaining of bronchial biopsies obtained from asthmatic subjects with the A_1 receptor antibody consistently revealed strong positive staining on the bronchial epithelium and moderate to strong positive staining on the bronchial smooth muscle, these distinctive regions being identified morphologically (Figure 1, panels A-C). The immunostaining appeared to be distributed uniformly throughout the whole of these regions, rather than localised to specific areas or structures within these regions. By comparison, immunostaining of bronchial biopsies obtained from healthy subjects with the A_1 receptor antibody was strikingly less intense, with weak positive staining of the epithelium and very weak or sometimes undetectable positive staining of the smooth muscle (Figure 2, panels A-C). In all asthmatic and healthy bronchial biopsy sections, a negligible level of non-specific staining was consistently observed with the isotype control antibody (data not shown).

Sections obtained from 11 asthmatic subjects and 7 healthy subjects only were deemed of a suitable quality for image analysis as sections obtained from one subject in each study group lacked sufficient epithelial and smooth muscle areas required for the analysis. Quantification of the staining intensity of the epithelium revealed that the level of positive staining of the asthmatic epithelium was significantly more intense than that observed with the healthy epithelium (175.2 ± 5.3 A.U. vs 78.9 ± 30.9 A.U., $P < 0.01$). Similarly, the intensity of the immunostaining of the asthmatic bronchial smooth muscle was significantly greater than the immunostaining observed with the healthy bronchial smooth muscle (132.3 ± 9.9 A.U. vs 58.1 ± 24.4 A.U., $P < 0.01$) (Figure 3).

Submucosal glands were identified on sections from two asthmatic bronchial biopsies and one healthy biopsy. Again, A_1 receptor expression appeared to be greater in the asthmatic biopsies (Figure 4, panel A), in comparison to the healthy biopsy (Figure 4, panel B).

Both the brain cortex and cardiac tissue samples from all three subjects immunostained very strongly for the A_1 receptor (illustrated in Figure 5, panels A and C respectively), with no detectable staining observed with the isotype control antibody (Figure 4, panels B and D). A small degree of brown colouration observed in the cardiac tissue (panel D) was considered to be due to the presence of lipofuscin pigments.

A_1 receptor antibody validation

In order to provide additional support to the observations resulting from the immunohistochemical analysis described above, antibody validation experiments were also performed to analyse the affinity and selectivity of the A_1 receptor antibody for the A_1 receptor.

Quantitative RT-PCR of the CHO-K1 cell lines transfected with one of the four adenosine receptor subtypes revealed that each cell line expressed very high transcript levels of the transfected receptor and that the gene expression levels of each receptor were generally of a similar magnitude (Figure 6), although the level was slightly lower in the A_3 cell line. Subsequent flow cytometric analysis of the CHO-K1 cell lines using the A_1 receptor antibody resulted in a significant increase in the mean fluorescence intensity when compared to wild type CHO-K1 cells only in the A_1 cell line (478 A.U. ± 84 vs 35 A.U. ± 8; $P < 0.01$). There were no significant differences between any of the other cell lines and the wild type cells (Figure 6).

DISCUSSION

Although the expression and functions of adenosine receptors have been studied in individual cell types, usually obtained from healthy subjects, there is very little information describing the distribution and relative expression of the four adenosine receptor subtypes in healthy or asthmatic airways. The aim of the present study therefore, was to investigate the distribution of A_1 receptors in bronchial biopsies obtained from mild asthmatic subjects and then compare the level of expression with biopsies taken from healthy subjects. Our data reveal that there is a low level of A_1 receptor expression present in healthy bronchial tissue, which appears to be predominantly located to the bronchial epithelium, whilst A_1 receptor expression is markedly elevated in asthmatic bronchial tissue, particularly on the bronchial epithelium and bronchial smooth muscle. Whilst the precise location of this receptor was not determined it is possible that this receptor is expressed on ciliated epithelial cells [15], goblet cells [16], sensory nerves [17], and smooth muscle cells [18]. Future studies using double labelling and confocal microscopy will discern the cell types involved.

In an attempt to quantify the increased expression in asthmatic tissue, we performed image analysis on epithelial and smooth muscle areas on one tissue section from each biopsy. Image analysis thus revealed that the mean intensity of the staining in these regions of asthmatic tissue was significantly greater than that observed with healthy tissue, which therefore confirmed higher A_1 receptor expression.

The A_1 receptor antibody used in this study was raised against a C-terminal portion of the A_1 receptor and was designed in order to limit the recognition of the other adenosine receptor subtypes by the antibody. To validate this antibody, experiments

were performed to provide evidence that the A_1 receptor antibody was able to selectively bind A_1 receptors. Flow cytometric analysis of CHO-K1 cells transfected with human recombinant adenosine receptors showed a significant increase in fluorescence only with the cell line expressing A_1 receptors when compared to wild type cells following incubation with the A_1 receptor antibody. The caveat to this is the assumption that there is similar adenosine receptor protein expression in each cell line. Although a formal comparison of protein expression was not attempted in the present study, an analysis of the mRNA transcript levels revealed similar levels of gene expression in each cell line used in our experiments and though the A_3 receptor transcript level was slightly lower than the other subtypes, the level was still of a magnitude to expect a very high degree of actual receptor protein expression. Further confirmation that our antibody recognises and binds to the A_1 receptor was demonstrated by a high level of positive immunostaining on the brain cortex and cardiac tissue samples, tissues which are recognised to have a high expression of the A_1 receptor. Therefore, having validated the selectivity of the A_1 receptor antibody, we conclusively then demonstrated for the first time that the A_1 receptor is markedly upregulated in bronchial tissue from subjects with asthma who are responsive to AMP challenge, and confirmed that the difference was a consistent observation with image analysis. Given the limitations of immunohistochemistry and image analysis, we would expect that the image analysis data are certainly not proportionally representative of total A_1 receptor number but simply indicate that there is a difference in the pattern of A_1 receptor expression between the mild asthmatic and healthy phenotypes.

Our observations support a number of studies investigating adenosine receptor expression in animal models of allergic lung inflammation, which also identified an increase in airway A_1 receptor expression when compared to healthy naïve animals [8, 19-22]. In a rabbit model of allergic lung inflammation, it was demonstrated that the A_1 receptor was upregulated on the airway smooth muscle and a functional consequence of this increase was that it mediated bronchoconstriction following inhalation of adenosine [8, 23]. Given our observations, we could therefore speculate that an increase in A_1 receptors on bronchial smooth muscle in atopic mild asthmatic subjects may contribute to the bronchoconstrictor response to AMP observed in these subjects. In support of this possibility, an investigation into the spasmogenic effect of adenosine on isolated human bronchial strips showed a variable but overall small contractile response in tissue from healthy subjects and that the magnitude of this was significantly increased in tissue from asthmatic subjects [12]. The adenosine-induced contraction in isolated tissue from asthmatic subjects was significantly inhibited by the A_1 receptor antagonist 2-thio-[(1,3-dipropyl)-8-cyclopentyl]-xanthine (DPCPX). The same study also showed that the response could be blocked by preincubation with an H_1 antagonist and leukotriene synthesis inhibitor, suggesting that the response was mast cell mediated. However, a subsequent investigation demonstrated that isolated human bronchial tissue has a high degree of intrinsic tone and that histamine and leukotrienes constitute a major part of this basal tone [24]. Thus, it is difficult to ascertain to what degree the inhibition of adenosine-induced contractile responses by the H_1 receptor antagonist and leukotriene synthesis inhibitor in the former study can be attributed to the inhibition of mast cell products released by adenosine, or simply a reduction in basal tone. Hence, further studies investigating the effects of selective adenosine receptor agonists and antagonists on isolated asthmatic bronchial tissue are

required to provide further insight into A_1-mediated contraction of bronchial smooth muscle obtained from subjects with asthma.

On a cellular level, however, an investigation into adenosine receptor signalling pathways in isolated airway smooth muscle cells from healthy donors identified A_{2B} receptors as the predominant adenosine receptor subtype [25]. The A_1 receptor is the subtype with the highest affinity for adenosine (reviewed in 26), so we could speculate that on asthmatic bronchial smooth muscle cells, adenosine may preferentially activate the upregulated A_1 receptor. Since the A_1 receptor is G_i-protein coupled, stimulation of this receptor would result in a decrease in cAMP, leading to smooth muscle contraction. In support of this, it has recently been demonstrated that activation of the A_1 receptor with a selective A_1 receptor agonist on healthy human airway smooth muscle cells *in vitro* induces calcium mobilisation [18]. Thus, given that H_1 receptor antagonists do not completely block AMP-induced bronchoconstriction in asthmatic subjects and co-administration of inhibitors of other mast cell products are not additive (reviewed in 4), it is likely that other mechanisms besides mast cell degranulation are involved in the response to AMP. Further studies are thus required to investigate these other mechanisms, including the potential direct spasmogenic effects of adenosine in asthmatic subjects.

The functional significance of the increased expression of the A_1 receptor on the asthmatic bronchial epithelium remains to be established. However, a recent study demonstrated that activation of the A_1 receptor on tracheal epithelial cells resulted in an increase in mucin gene expression [16]. Our observation of an increase in A_1 receptor expression on the bronchial epithelium in asthmatic subjects thus further

implicates adenosine in promoting mucus hyperscretion *via* activation of the A_1 receptor. Furthermore, in tissue sections from three subjects, submucosal glands were identified. Two were from asthmatic subjects and high A_1 receptor expression was observed yet was not apparent in sections from the healthy subject, but these are only preliminary observations and further investigations are required before definitive conclusions can be made. However, adenosine has been shown to induce canine tracheal mucus secretion *in vivo* through an A_1 receptor dependent mechanism [27], supporting the notion of adenosine as a secretagogue.

Interestingly, it has very recently been demonstrated that subjects with chronic obstructive pulmonary disease (COPD), who also have an increased extracellular level of adenosine in the airways, have an increased density of A_1, A_{2A} and A_3 receptors in the lung parenchyma when compared to age-matched smoking controls [28]. However, in order to investigate the role of adenosine in asthma further, our future studies will aim to characterise the expression of the other adenosine receptor subtypes in asthmatic subjects, in addition to investigating the effects of glucocorticosteroid treatment on adenosine receptor expression, since clinically glucocorticosteroids decrease hyperresponsiveness to AMP in subjects with asthma [29].

Our evidence of an increased adenosine A_1 receptor expression in asthmatic subjects reflects studies characterising adenosine receptor expression in mice partially lacking adenosine deaminase [30]. These mice accumulate high levels of adenosine extracellularly and exhibit severe pulmonary inflammation. Some of the pathological features reported in this model were consistent with those observed in asthma such as

mucus hypersecretion, but the pulmonary inflammatory cell profile differed considerably and consisted mainly of macrophages, with no increase in eosinophil numbers. However, transcript levels for the A_1, A_{2B} and A_3 receptors were found to be significantly increased, suggesting that an increase in extracellular adenosine promotes adenosine receptor signalling. The pulmonary inflammation in mice totally deficient in adenosine deaminase was even more severe and the mice died at three weeks of age from respiratory distress [31]. Interestingly, concurrent knockout of the adenosine A_1 receptor was reported to exacerbate the inflammation further, suggesting a protective role of the A_1 receptor in this model [32]. However, the levels of eosinophils in this model were of a magnitude unlikely to result in alterations in airways responsiveness and therefore the relevance of these findings to asthma should be interpreted with caution (less than 2% of total cells, compared with approximately 40% of total in a murine model of allergic inflammation [33]).

In conclusion therefore, we have demonstrated for the first time that adenosine A_1 receptor expression is markedly upregulated in bronchial tissue obtained from subjects with asthma who are responsive to AMP challenge, particularly on bronchial smooth muscle and the airway epithelium.

Acknowledgements

We thank David Hall and Ken Saunders for technical assistance with flow cytometry.

References

1. Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 2004: 25: 33-39.

2. Driver AG, Kukoly CA, Ali S, Mustafa, S.J. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 1993: 148: 91-97.

3. Huszar E, Vass G, Vizi E, Csoma Z, Barat E, Molnar Vilagos G, Herjavecz I. Horvath, I. Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *Eur Respir J* 2002: 20:1393-1398.

4. Meade CJ, Dumont I, Worrall L. Why do asthmatic subjects respond so strongly to inhaled adenosine? *Life Sci* 2001: 69: 1225-1240.

5. Forsythe P, Ennis M. Adenosine, mast cells and asthma. *Inflamm Res* 1999: 48: 301-307.

6. van den Berge M, Kerstjens HA, de Reus DM, Koeter GH, Kauffman HF, Postma DS. Provocation with adenosine 5'-monophosphate, but not methacholine, induces sputum eosinophilia. *Clin Exp Allergy* 2004: 34: 71-76.

7. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl Physiol* 1986: 61:1667-1676.

8. Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997: 385: 721-725.

9. Obiefuna PC, Batra VK, Nadeem A, Borron P, Wilson CN, Mustafa SJ. A Novel A_1 Adenosine Receptor Antagonist (L-97-1) Reduces Allergic Responses to House Dust Mite in an Allergic Rabbit Model of Asthma. *J Pharmacol Exp Ther* 2005: 315: 329-336.

10. Vizi E, Huszar E, Csoma Z, Boszormenyi-Nagy G, Barat E, Horvath I, Herjavecz I, Kollai M. Plasma adenosine concentration increases during exercise: a possible contributing factor in exercise-induced bronchoconstriction in asthma. *J Allergy Clin Immunol* 2002: 109: 446-448.

11. Csoma Z, Huszar E, Vizi E, Vass G, Szabo Z, Herjavecz I, Kollai M, Horvath I. Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction. *Eur Respir J* 2005: 25: 873-878.

12. Bjorck T, Gustafsson LE, Dahlen SE. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am Rev Respir Dis* 1992: 145: 1087-1091.

13. Joad JP. Characterization of the human peripheral lung adenosine receptor. *Am J Respir Cell Mol Biol* 1990: 2:193-198.

14. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997: 16: 531-537.

15. McCoy DE, Schwiebert EM, Karlson KH, Spielman WS, Stanton BA. Identification and function of A_1 adenosine receptors in normal and cystic fibrosis human airway epithelial cells. *Am J Physiol* 1995: 268(6 Pt 1): C1520-1527.

16. McNamara N, Gallup M, Khong A, Sucher A, Maltseva I, Fahy J, Basbaum C. Adenosine up-regulation of the mucin gene, *MUC2*, in asthma. *FASEB J* 2004: 18: 1770-1772.

17. Chuaychoo B, Lee MG, Kollarik M, Pullmann R, Undem BJ. Evidence for both adenosine A_1 and A_{2A} receptors activating single vagal sensory C-fibres in guinea pig lungs. J Physiol 2006: 575(Pt 2):481-490.

18. Ethier MF, Madison JM. Adenosine A_1 receptors mediate mobilization of calcium in human bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 2006: 35: 496-502.

19. Ali S, Mustafa SJ, Metzger WJ. Adenosine receptor-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbit model. *Am J Physiol* 1994: 266: L271-277.

20. Ali S, Mustafa SJ, Metzger WJ. Adenosine-induced bronchoconstriction and contraction of airway smooth muscle from allergic rabbits with late-phase airway obstruction: evidence for an inducible adenosine A_1 receptor. *J Pharmacol Exp Ther* 1994: 268: 1328-1334.

21. el-Hashim A, D'Agostino B, Matera MG, Page C. Characterization of adenosine receptors involved in adenosine-induced bronchoconstriction in allergic rabbits. *Br J Pharmacol* 1996: 119: 1262-1268.

22. Pauwels RA, Joos GF. Characterization of the adenosine receptors in the airways. *Arch Int Pharmacodyn Ther* 1995: 329: 151-160.

23. Abebe W, Mustafa SJ. A_1 adenosine receptor-mediated Ins(1,4,5)P3 generation in allergic rabbit airway smooth muscle. *Am J Physiol* 1998: 275: L990-997.

24. Ellis JL, Undem BJ. Role of cysteinyl-leukotrienes and histamine in mediating intrinsic tone in isolated human bronchi. *Am J Respir Crit Care Med* 1994: 149: 118-122.

25. Mundell SJ, Olah ME, Panettieri RA, Benovic JL, Penn RB. Regulation of G protein-coupled receptor-adenylyl cyclase responsiveness in human airway smooth muscle by exogenous and autocrine adenosine. *Am J Respir Cell Mol Biol* 2001: 24: 155-163.

26. Marx D, Ezeamuzie CI, Nieber K, Szelenyi I. Therapy of bronchial asthma with adenosine receptor agonists and antagonists. *Drug News Perspect* 2001: 14: 89-100.

27. Johnson HG, McNee ML. Adenosine-induced secretion in the canine trachea: modification by methylxanthines and adenosine derivatives. *Br J Pharmacol* 1985: 86: 63-67.

28. Varani K, Caramori G, Vincenzi F, Adcock I, Casolari P, Leung E, Maclennan S, Gessi S, Morello S, Barnes PJ, Ito K, Chung KF, Cavallesco G, Azzena G, Papi A, Borea PA. Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006: 173: 398-406.

29. Spicuzza L, Bonfiglio C, Polosa R. Research applications and implications of adenosine in diseased airways. *Trends Pharmacol Sci* 2003: 24: 409-413.

30. Chunn JL, Young HWJ, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J Immunol* 2001: 167: 4676-4685.

31. Blackburn MR, Volmer JB, Thrasher JL, Zhong H, Crosby JR, Lee JJ, Kellems RE. Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction. *J Exp Med* 2000: 192: 159-170.

32. Sun CX, Young HW, Molina JG, Volmer JB, Schnermann J, Blackburn MR. A protective role for the A_1 adenosine receptor in adenosine-dependent pulmonary injury. *J Clin Invest* 2005: 115: 35-43.

33. Riffo-Vasquez Y, Spina D, Page C, Tormay P, Singh M, Henderson B, Coates A. Effect of Mycobacterium tuberculosis chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* 2004: 34: 712-719.

Tables

Table I Patient demographics

	Asthmatic	Healthy
Number	16	8
Sex (male:female)	11:5	4:4
Age (yr)	26.6 (20-36)*	25.6 (20-37)*
Baseline FEV_1 (%predicted)	92.7 (74-120)*	106.8 (89-127)*
Height (cm)	175.2 (160-186)*	179 (159-188)*
Weight (Kg)	74 (56-95)*	79.2 (52-106)*
AMP PC_{20} (mg ml^{-1})	28.1 (0.88-136)*	N/A
Methacholine PC_{20} (mg ml^{-1})	1.13 (0.29-2.88)	>16 mg ml^{-1}

*Geometric mean (range)

Figure legends

Figure 1

Photomicrographs representative of positive immunostaining of asthmatic bronchial biopsy sections with an A_1 receptor antibody. Positive immunostaining has a brown appearance against a blue background resulting from Mayer's haematoxylin counterstain. Panel A (X 200) shows high expression of the A_1 receptor on the epithelium and smooth muscle, the same section is also shown in panels B and C respectively at 400 X magnification, in order to see the epithelium (B) and smooth muscle (C) more clearly.

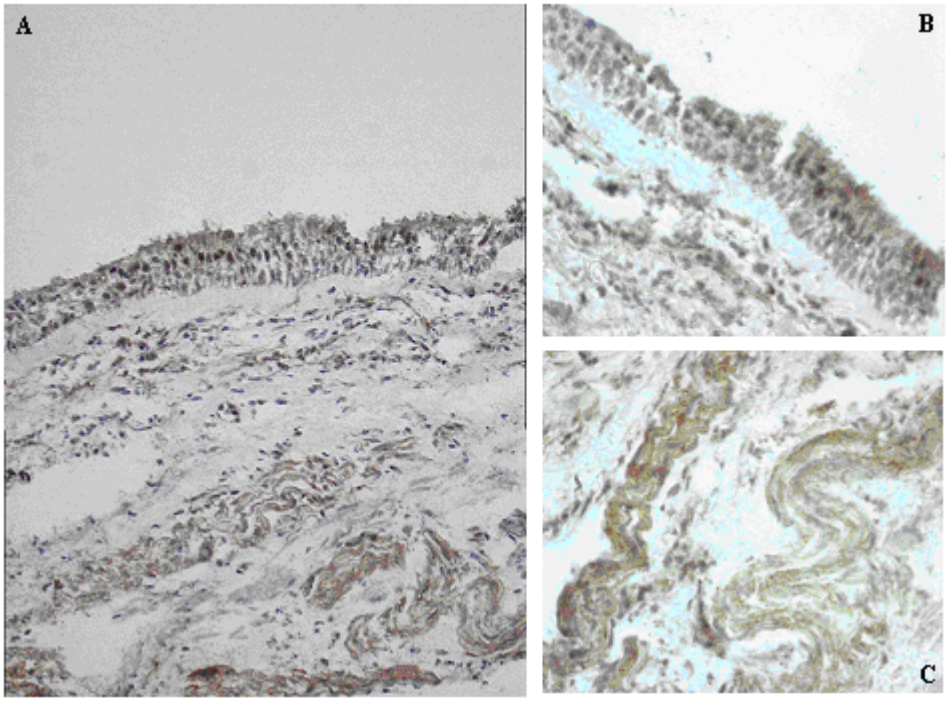

A
B
C

Figure 2

Photomicrographs representative of positive immunostaining of healthy bronchial biopsy sections with an A_1 receptor antibody. Positive immunostaining has a brown appearance against a blue background resulting from Mayer's haematoxylin counterstain. Panel A (X 200) shows weak expression of the A_1 receptor on the epithelium and virtually no positive immunostaining of smooth muscle. Panels B and C show the epithelium and smooth muscle respectively at 400 X magnification for clarity.

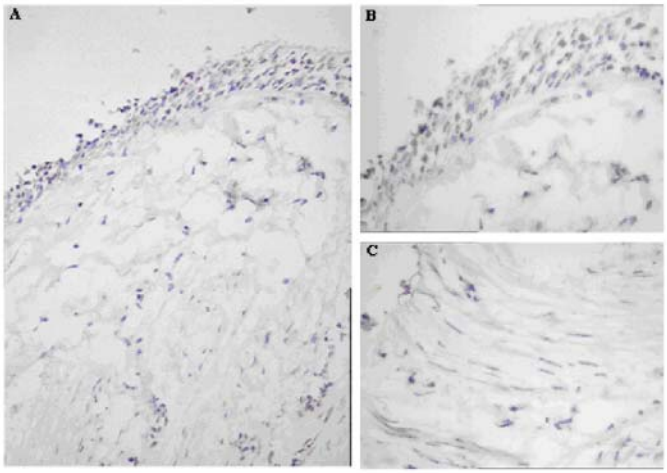


Figure 3

Image analysis of asthmatic and healthy bronchial biopsies immunostained with an A_1 receptor antibody. The intensity of positive staining specifically on the epithelium and smooth muscle on bronchial biopsy sections from 11 asthmatic subjects and 7 healthy subjects (1 section per subject) was quantified using Zeiss Vision KS400 software, from 4 different views of both epithelium and smooth muscle at 1000X magnification. Data are expressed as the mean of intensity staining ± SEM, **$P < 0.01$ and $^{\#\#}P < 0.01$.

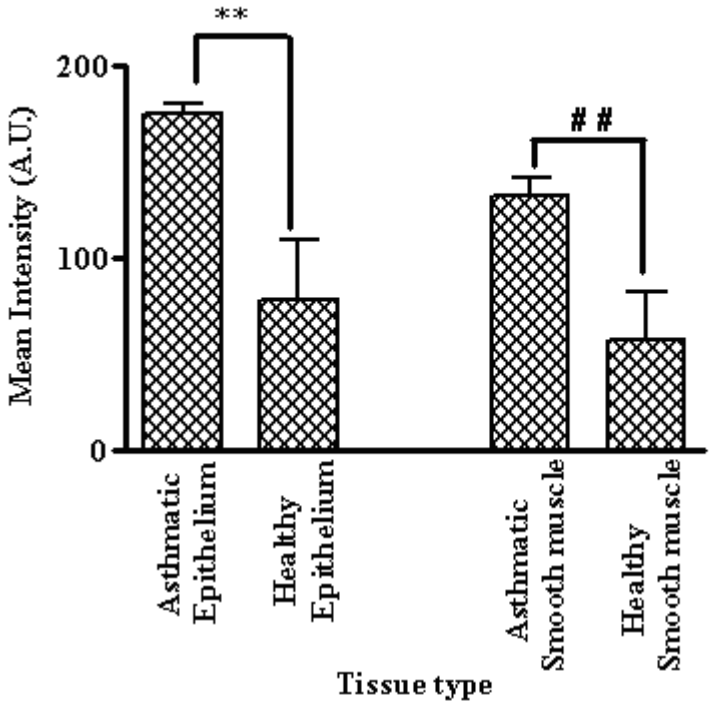

**
# #
200
100
0
Mean Intensity (A.U.)
Asthmatic Epithelium
Healthy Epithelium
Asthmatic Smooth muscle
Healthy Smooth muscle
Tissue type

Figure 4

Photomicrographs representative of immunostaining of asthmatic and healthy bronchial biopsy sections with an A_1 receptor antibody. Positive immunostaining has a brown appearance against a blue background resulting from Mayer's haematoxylin counterstain. Panel A (X 400) shows high expression of the A_1 receptor on the submucosal glands, panel B (X 400) shows no apparent positive immunostaining of healthy submucosal glands.

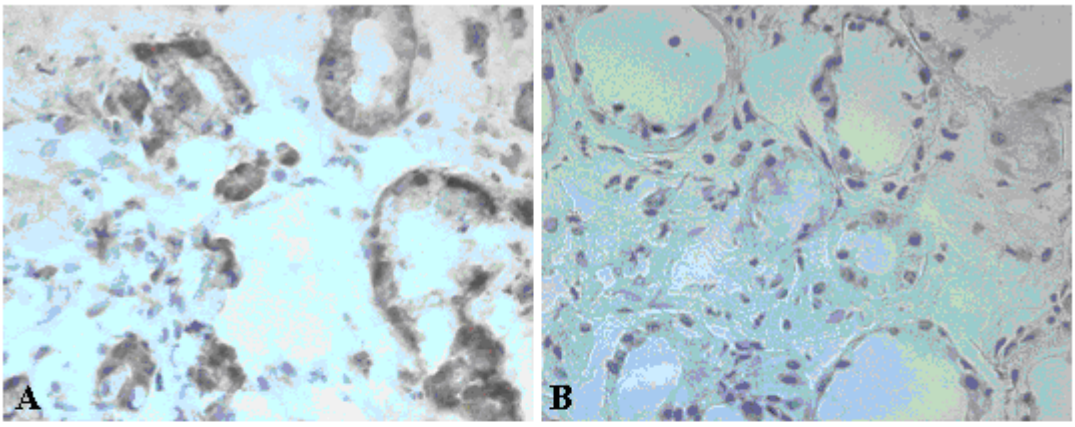


Figure 5

Photomicrographs (X 200) illustrating positive staining of brain cortex and cardiac tissue with the A_1 antibody (panels A and C respectively). Positive immunostaining is identified by a brown appearance against a blue background resulting from Mayer's haematoxylin counterstain. Panels B and D respectively show the negligible staining of brain cortex and cardiac sections from the same samples following staining with an isotype control antibody.

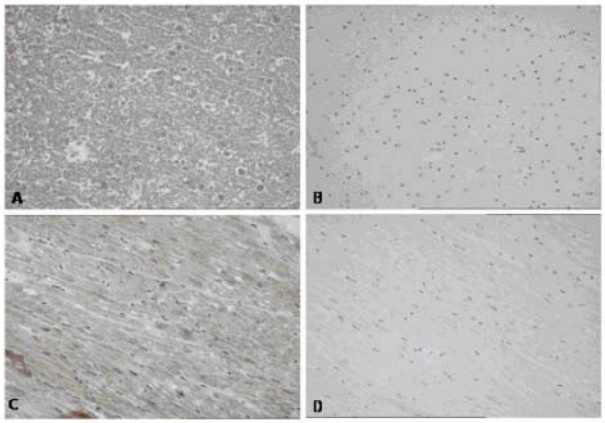
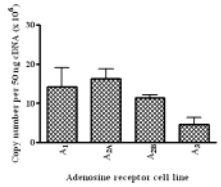
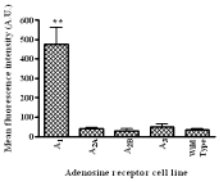

A
B
C
D

Figure 6

Quantitative real-time RT-PCR (A) and flow cytometric analysis (B) of CHO-K1 cells transfected with the human recombinant adenosine A_1, A_{2A}, A_{2B} or A_3 receptor. Expression of each transfected receptor was analysed using the appropriate primer pair and probe described in Table II in the online supplementary material. PE-labelled cells were analyzed by appropriate gating for immunofluorescence after excitation at 488 nm. At least 3,000 events were collected and mean fluorescence intensity values recorded. Results are from 3 separate experiments and data are expressed as mean copy number per 50 ng cDNA ± SEM (A) and mean fluorescence intensity (A.U. ± SEM) (B) respectively. An increase in fluorescence is observed only in the A_1 transfected cell line (**$P < 0.01$ when compared to wild type cells).


Copy number per 50 ng cDNA (x 10^6)
30
20
10
0
A1
A2A
A2B
A3
Adenosine receptor cell line


Mean fluorescence intensity (A.U.)
600
500
400
300
200
100
0
**
A1
A2A
A2B
A3
Wild Type
Adenosine receptor cell line