

Airway inflammation and hyperresponsiveness to adenosine 5'-monophosphate in chronic obstructive pulmonary disease

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Summary

Background Chronic obstructive pulmonary disease (COPD) is often accompanied by bronchial hyperresponsiveness (BHR). Measurement of BHR may give information about airway inflammation.

Objective To investigate the role of airway inflammation in hyperresponsiveness to adenosine 5'-monophosphate (AMP) in COPD.

Methods We investigated inflammatory indices in induced sputum, bronchoalveolar lavage (BAL) fluid and bronchial biopsies in subjects with COPD with and without hyperresponsiveness to AMP. Twelve nonatopic subjects with COPD with hyperresponsiveness to AMP (mean $\pm$ SD, age 63 ± 8 years, FEV₁% predicted $56 \pm 13\%$), six without BHR (age 60 ± 6 years, FEV₁% predicted $65 \pm 11\%$) and 11 healthy nonatopic controls without BHR (age 58 ± 8 years, FEV₁% predicted $104 \pm 11\%$) participated in the study.

Results Subjects with COPD with BHR had significantly higher numbers of mucosal CD8+ and higher percentages of sputum eosinophils than those without BHR (median, 550 cells/mm²; range, 30–1340 vs 280 cells/mm²; range, 110–450, $P = 0.045$; and median, 2.7%; range, 0.5–8.5 vs 0.6%; range, 0–0.8 %, $P = 0.0036$, respectively). No differences were observed in BAL fluid.

Conclusion We conclude that hyperresponsiveness to AMP in COPD is associated with airway inflammation that is characterized by increased numbers of mucosal CD8+ cells and percentages of sputum eosinophils. Hyperresponsiveness to AMP may be used as a marker of airway inflammation in COPD, but its significance in the clinical course remains to be determined.

Keywords: adenosine 5'-monophosphate, BAL, biopsy, COPD, hyperresponsiveness, inflammation, sputum

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Introduction

Chronic obstructive pulmonary disease (COPD) is a slowly progressive disease with a reduction of the forced emptying of the lungs that does not change markedly over several months [1]. Hyperresponsiveness is often present and is determined by airway diameter [2], airway wall thickening and loss of lung tissue elastic recoil [3], and possibly by

airway inflammation as well [4,5]. Hyperresponsiveness can be tested with stimuli that act directly on airway smooth muscle cells and with stimuli that cause airway smooth muscle contraction via an indirect mechanism [6]. Indirect stimuli, e.g. adenosine 5'-monophosphate (AMP), have been suggested to indicate airway inflammation better than direct stimuli [7].

AMP can induce bronchoconstriction in subjects with COPD, whereas it does not in healthy controls [8]. Airway inflammation may play a role in AMP-induced bronchoconstriction, since hyperresponsiveness to AMP has a

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higher prevalence and severity in smokers than in exsmokers with COPD with comparable hyperresponsiveness to methacholine [8]. However, short-term anti-inflammatory treatment failed to improve hyperresponsiveness [9,10]. Mast cells but not cholinergic nerves seem to be involved in AMP-induced bronchoconstriction in COPD, since terfenadine decreased hyperresponsiveness, whereas ipratropium bromide failed [11].

In order to better understand the role of airway inflammation in hyperresponsiveness to AMP in COPD, we investigated induced sputum, bronchoalveolar lavage (BAL) fluid and bronchial biopsies in subjects with COPD with and without hyperresponsiveness to AMP.

Methods

Subjects

Twenty subjects with COPD, according to American Thoracic Society criteria [12], and 12 healthy control subjects participated in the study. Subjects were exsmokers or nonsmokers. Subjects with COPD had a forced expiratory volume in 1 sec (FEV₁) and FEV₁/vital capacity (VC) < predicted value – 1.64 residual SD [6] and an increase in FEV₁ of < 10% predicted after inhalation of 1 mg of terbutaline per Turbuhaler®. All subjects had a negative history of atopy, negative skin tests to 18 common aeroallergens (ALK, Groningen, The Netherlands), and negative specific serum IgE for 11 common aeroallergens. Thirteen subjects who had maintenance treatment with inhaled corticosteroids discontinued this at least 1 month prior to the study. Exclusion criteria were treatment with oral corticosteroids and antibiotics or a respiratory tract infection in the month prior to the study. Healthy volunteers did not have a history of pulmonary disease, had normal lung function, were nonhyperresponsive to AMP and were matched for age and pack years. The protocol was approved by the hospital ethical committee. All subjects gave their informed consent.

Design

The study involved four visits. At the first visit, history was taken, physical examination was performed, and spirometry with reversibility test and atopy were assessed. At the second visit, a challenge with AMP was done. At the third visit, 7–14 days later, sputum was induced. At the fourth visit, 7–14 days later, a bronchoscopy with BAL and bronchial biopsies was performed.

Spirometry and AMP challenge

Spirometry was performed using a calibrated water-sealed spirometer (Lode BV, Groningen, The Netherlands)

according to standardized guidelines [6]. Doubling doses of AMP (range 0.04–320 mg/mL) were administered according to a 2-min tidal breathing protocol, as described elsewhere [9].

Sputum induction

Sputum was induced via inhalation of hypertonic saline aerosol, generated by an ultrasonic nebulizer (Ultraneb 2000, DeVilbiss, Somerset, Pennsylvania, USA). After inhalation of 1 mg of terbutaline, solutions of sodium chloride of 3%, 4% and 5% (w/v) were nebulized at room temperature during 7-min period. Subjects cleared their mouth and throat with water and expectorated sputum into a sterile plastic container, which was kept on ice. The procedure was terminated after three periods of 7 min or after a fall in FEV₁ of 20% or more from baseline value.

Sputum processing

Sputum was processed within 15 min after termination of the induction. The whole sample was homogenized after addition of an equal volume of dithiothreitol 0.1% (Sputolysin; Calbiochem, La Jolla, CA, USA) and filtered, followed by total cell count and viability check by trypan blue exclusion. The filtered sample was centrifuged and the supernatant was aspirated and stored at –80 °C. Two cytopsin slides for differential cell counts were made and stained with May-Grünwald-Giemsa (MGG). Differential cell counts were performed by counting 300 nonsquamous cells in each coded MGG cytopsin in a blind way by two technicians.

Bronchoscopy

The flexible fiberoptic bronchoscope (Olympus B1 IT10, Olympus Optical, Tokyo, Japan) was introduced wedged in a subsegmental bronchus of the middle lobe. Lavage was performed with four 50-mL aliquots of normal saline prewarmed at 37 °C. The first aliquot was recovered separately, the following three aliquots were pooled and used for analysis. After completion of lavage, bronchial biopsies were taken from subsegmental carinae of the right or left lower lobe using cup forceps (Olympus BF-21C, Tokyo, Japan).

Lavage processing

BAL fluid was placed on ice and processed within 10 min after recovery. BAL fluid was filtered and recovery was assessed. BAL fluid was centrifuged and supernatant was stored at –80 °C. The cell pellet was resuspended for total

Table 1. Clinical characteristics of subjects

	COPD		Healthy
	AMP-responsive (<i>n</i> = 12)	AMP-nonresponsive (<i>n</i> = 6)	AMP-nonresponsive (<i>n</i> = 11)
PC ₂₀ AMP, mg/mL†	21.56 (1.08–267.23)*	> 320	> 320
Age (years)	63 ± 8	60 ± 6	58 ± 8
Smoking (pack years)	27 ± 21	29 ± 21	25 ± 15
Years after quit smoking	11 ± 12	11 ± 14	15 ± 10
Nonsmoker	2	1	0
FEV ₁ , % predicted	56 ± 13*	65 ± 11*	104 ± 11
FEV ₁ , % predicted	6 ± 2*	5 ± 3	3 ± 3
Gender, M/F	9/3	5/1	8/3
Serum eosinophils, n/mL†	200 (30–540)*	120 (60–350)	91 (13–430)
Sputum induction, <i>n</i>	12	6	11
Bronchoalveolar lavage, <i>n</i>	10	6	11
Bronchial biopsy, <i>n</i>	12	5	10

† Mean ± SD or geometric means (ranges). **P* < 0.05, compared with healthy subjects. FEV₁: forced expiratory volume in 1 sec. PC₂₀AMP: provocative concentration of AMP causing a 20% decrease in FEV₁.

cell count, viability assessment and cytopins (see *sputum processing*). Cell counting was performed as in induced sputum.

Biopsy processing and cell quantification

The biopsies were embedded in Tissue Tek® (Bayer Corporation, Elkhart, IN, USA), and snap-frozen in liquid isopentane (–80 °C). Frozen biopsies were cut into sections of 4 µm thickness. Tissue slides were air-dried for 20 min and fixed in acetone for 10 min before staining. Immunohistochemical staining was performed with monoclonal antibodies against CD3 and CD4 (Beckton Dickinson, San Jose, USA), CD8 (mouse monoclonal antibody from our own laboratory), CD68 (Dako, Copenhagen, Denmark), secreted form of eosinophil cationic protein (ECP and EG2) (Sanbio, Uden, the Netherlands), neutrophil elastase NP57 (Dako), and mast cell tryptase AA1 (Dako). As a control, the immunohistochemical procedure was performed without the primary antibody and by replacing the primary antibody by a species and isotype-matched non-relevant antibody. Labelling of first antibodies was performed by biotinylated rabbit antimouse immunoglobulin antiserum and subsequently streptavidin conjugated to horseradish peroxidase. Amino-ethyl carbazole (AEC) was used as a chromogen, hydrogen peroxide for blocking of endogenous peroxidase and haematoxylin as a counterstain. For each antibody, the number of positively stained cells was counted in a zone of 100 µm deep below a randomly selected area of 1 mm of epithelial basement membrane.

Slides were coded and counted by one observer, using a Zeiss light microscope, in a blinded fashion.

Biochemical assays

The concentration of ECP in sputum supernatant and BAL fluid was measured using the fluoroenzyme immunoassay, ImmunoCAP ECP, kindly provided by Pharmacia, Uppsala, Sweden.

Statistics

Clinical characteristics are presented as means ± SD unless stated otherwise, cell and mediator characteristics as medians with ranges. Differences between subjects with COPD and healthy subjects and within the COPD group were tested with the Mann–Whitney *U*-test. Correlation between cell variables were analysed using the Spearman's rank correlation coefficient. Differential sputum and BAL fluid cell counts were expressed as percentages of nonsquamous cells. A *P*-value < 0.05 was considered statistically significant.

Results

Subjects

Two subjects with COPD did not yield sufficient BAL fluid and biopsies for differential cell counting and were excluded from the analyses. One healthy subject was unwilling to

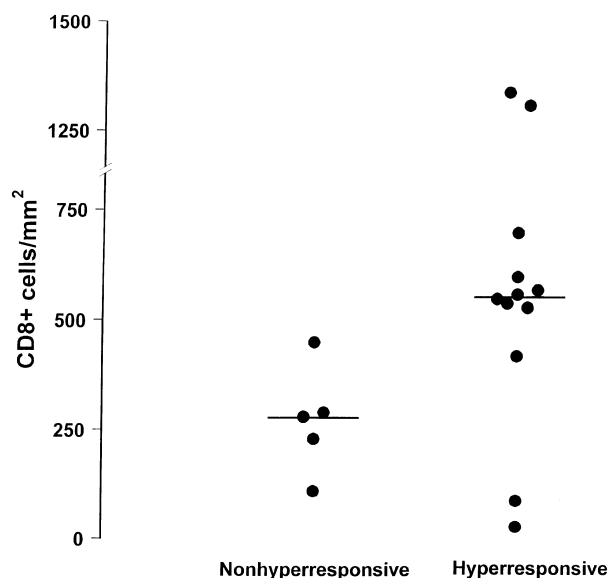


Fig. 1. Mucosal CD8+ cells in biopsies of subjects with COPD with or without hyperresponsiveness to adenosine 5'-monophosphate

undergo bronchoscopy. The clinical characteristics of the remaining 29 subjects are listed in Table 1. Twelve subjects with COPD had a decrease in FEV₁ of $\geq 20\%$ from baseline during AMP challenge. Six subjects with COPD and all 11 healthy subjects did not respond to AMP. Two subjects with COPD had cytospins in BAL fluid of too low quality for

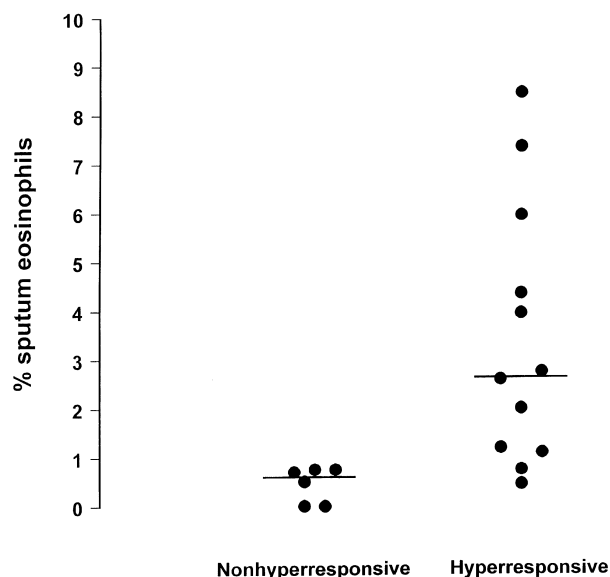


Fig. 2. Percentage of sputum eosinophils in biopsies of subjects with COPD with or without hyperresponsiveness to adenosine 5'-monophosphate

differential cell counting. One other subject with COPD and one healthy subject had biopsies of too low quality for cellular counting. There were no significant differences between the hyperresponsive and nonhyperresponsive subjects with COPD. Both groups had a significantly lower FEV₁ and those with hyperresponsiveness to AMP additionally had higher reversibility of FEV₁ and higher numbers of serum eosinophils compared with the healthy controls. Otherwise no significant differences were observed.

Biopsies

Subjects with hyperresponsiveness to AMP had more mucosal CD8+ cells than nonhyperresponsive patients with COPD (median, 550 cells/mm²; range 30–1340 vs 280 cells/mm²; 110–450, $P=0.045$) (Fig. 1). No other significant differences were observed between the two groups. The total group of subjects with COPD had significantly more mucosal CD68+ and EG2+ cells than the healthy subjects (median, 1115 cells/mm²; 330–2920 vs 590 cells/mm²; 450–1580, $P=0.03$; and 400 cells/mm²; 0–1900 vs 50 cells/mm²; 0–400, $P=0.049$, respectively).

Sputum

The median percentage of squamous cell contamination was 5% (range, 0–12) and 6% (range, 3–14) in subjects with COPD and healthy subjects, respectively. Subjects with hyperresponsiveness to AMP had higher percentages of eosinophils in the sputum than the nonhyperresponsive patients with COPD; 2.7% (range, 0.5–8.5) vs 0.6% (range, 0–0.8%, $P=0.0036$) (Fig. 2). The concentration of ECP per eosinophil was not significantly different between the two groups. The total group of subjects with COPD had significantly higher percentages of neutrophils and eosinophils and higher concentrations of ECP than the healthy subjects, median, 77% (range, 29–94) vs 36% (range, 18–69, $P=0.0001$); 1.2% (range, 0–8.5) vs 0.2% (range, 0–3.1, $P=0.0083$); and 838 ng/mL (range, 115–23 760) vs 121 ng/mL (range, 35–218), $P=0.0001$, respectively). The concentration of ECP per eosinophil was not significantly different between subjects with COPD and healthy subjects. ECP levels correlated significantly with the absolute number but not with the percentage of eosinophils in the whole group of COPD patients ($\rho=0.54$, $P=0.03$; and $\rho=0.35$, $P=0.16$, respectively).

BAL fluid

No significant differences in all available BAL parameters were observed between the two groups with COPD. The total group of subjects with COPD had significantly higher

percentages of eosinophils than the healthy subjects (0.4%; range, 0–1.7 vs 0.2%; range, 0–0.5; $P=0.03$). ECP was detectable in BAL fluid in only seven subjects with COPD; in these seven: median 12 ng/mL (range, 4–62), and not in healthy subjects

Discussion

We found that subjects with COPD with hyperresponsiveness to AMP have more mucosal CD8+ cells and higher percentages of sputum eosinophils than those without hyperresponsiveness. The association between hyperresponsiveness and increased numbers of mucosal CD8+ cells and percentages of sputum eosinophils may not simply be a causative one. We propose that the hyperresponsiveness and increased cell numbers and percentages are a manifestation of ongoing airway inflammation in these subjects with COPD who do not smoke (any more). The role of mucosal CD8+ cells and sputum eosinophils in COPD is not yet clear.

Interestingly, it has been shown recently that numbers of mucosal CD8+ are increased in smokers with COPD and correlate negatively with FEV₁ in smokers with chronic bronchitis [13]. The CD8+ T cells may contribute to tissue destruction via release of perforin or granzymes or via interference with normal tissue repair [14,15]. It remains to be established in how far CD8+ cells contribute causally to airway obstruction and hyperresponsiveness to AMP.

Sputum eosinophils do not seem to play an active role in ongoing inflammation in COPD, since the normal concentrations of ECP per eosinophil in both COPD groups suggest that these cells were not activated. The increased percentages of eosinophils may be a side effect of ongoing inflammation. Expression of adhesion molecules may be increased in ongoing airway inflammation [16], thereby causing increased nonspecific recruitment of eosinophils.

The identification of airway inflammation with sputum eosinophilia via hyperresponsiveness to AMP, however, might create therapeutic possibilities. In a previous study, we found no overall improvement of hyperresponsiveness to AMP in smokers with COPD after 6 weeks treatment with high-dose-inhaled corticosteroids compared with placebo [9]. However, after re-analysis of the data it appeared that there was a significant positive correlation between improvement in PC₂₀AMP and the level of peripheral blood eosinophils (Pearson's $r=0.53$, $P=0.013$) in subjects who received corticosteroids (personal observation). This may imply that airway inflammation in COPD, characterized by sputum and serum eosinophilia, is sensitive to corticosteroids and that a possible treatment effect can be assessed via hyperresponsiveness to AMP. Indeed, recent findings by Chanez *et al* [17] and Pizzichini *et al* [18] suggest that subjects with COPD who respond to

corticosteroids have increased percentages of eosinophils in induced sputum or BAL fluid. Keatings *et al* found no decrease in sputum eosinophil percentage and ECP concentration after 2 weeks of treatment with oral or inhaled glucocorticoids [19]. This lack of effect can be due to the short treatment period or may indicate that sputum eosinophils and ECP are not the right parameters to evaluate the short-term effect of corticosteroids. The finding of this study that sputum eosinophils are not activated but are rather the result of ongoing airway inflammation is in line with the latter view.

Increase of airway smooth muscle mass and loss of elastic recoil may partly have determined hyperresponsiveness in this study [3,20]. Our findings, however, suggest that airway inflammation may also play a role in hyperresponsiveness to AMP. It has been shown that histamine release by mast cells has only a limited role and vagal nerve activity is of little or no importance in AMP-induced bronchoconstriction [11], which implies that other inflammatory pathways may be important. Since smoking is an important aetiological factor of COPD [21] and it is associated with a higher frequency and severity of responsiveness to AMP in COPD [8], inflammatory changes caused by smoking may have a role in AMP-induced bronchoconstriction. Smoking can stimulate sensory nerve endings in the airway wall, which subsequently release tachykinins [22,23]. These tachykinins can cause smooth muscle contraction via binding to specific cell receptors and this is a possible pathway for the effects of inhalation of AMP in COPD. Tachykinins can enhance hyperresponsiveness via induction of airway wall oedema and mucus hypersecretion and possibly via induction of smooth muscle hypertrophy resulting from chronic stimulation. Moreover, they can induce recruitment of inflammatory cells into the airways. Therefore, tachykinins may be important in hyperresponsiveness to AMP in subjects with COPD.

We investigated the role of airway inflammation in hyperresponsiveness to AMP in COPD. In asthma, airway inflammation has been shown to be important in hyperresponsiveness to AMP [24]. Mast cell mediators and neural reflexes are mainly involved in AMP-induced bronchoconstriction in asthma [25,26]. Regular treatment with inhaled steroids resulted in a significantly greater reduction in AMP responsiveness than direct and neurally-acting stimuli [27]. The type and number of inflammatory cells in the airways differ between asthma and COPD [28]. Therefore, the mechanism of bronchoconstriction may differ between asthma and COPD, as has been shown by different studies [9,11,26,27].

Only few nonhyperresponsive patients with COPD participated in the study. Therefore, possible differences between the hyperresponsive and nonhyperresponsive group may not have been statistically significant. It is

possible that more manifestations of airway inflammation in induced sputum, BAL fluid and bronchial biopsies might have been detected with a larger group of patients.

We conclude that hyperresponsiveness to AMP in COPD is associated with airway inflammation, which is characterized by increased numbers of mucosal CD8+ cells and percentages of sputum eosinophils. We suggest further studies to unravel the role of tachykinins in AMP-induced bronchoconstriction in COPD and the role of hyperresponsiveness to AMP as a marker of airway inflammation in the management of COPD.

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