Indirect airways responsiveness and cell activation as inflammatory parameters in nocturnal asthma

Oosterhoff, Ytsche

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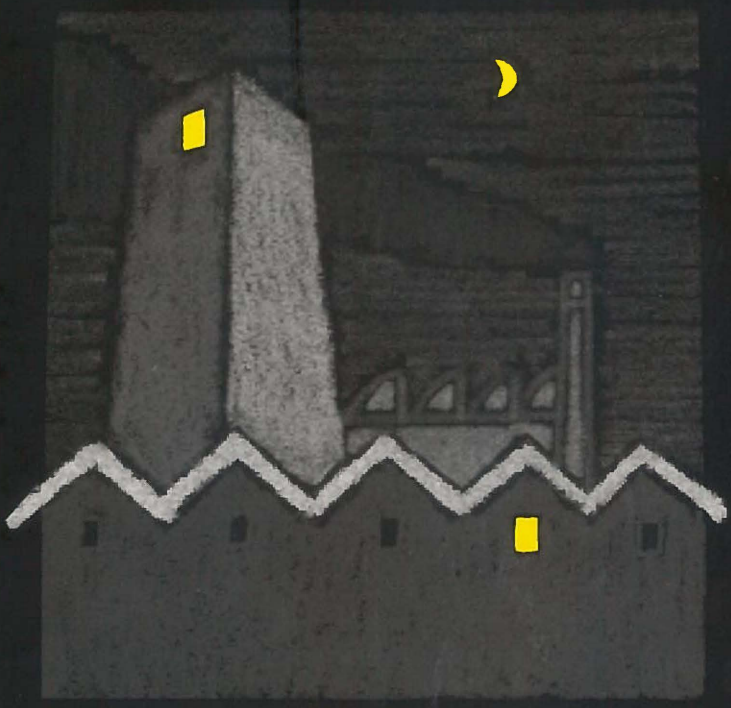

Ytsche Oosterhoff

INDIRECT AIRWAYS RESPONSIVENESS AND CELL ACTIVATION AS INFLAMMATORY PARAMETERS IN NOCTURNAL ASTHMA

STELLINGEN

De term 'nachtelijk astma' kan beter worden gewijzigd in 'astma met nachtelijke symptomen'.

De therapie van nachtelijk astma dient primair gericht te zijn op reduktie van ontstekingsaktiviteit in de luchtwegen.

Het meten van luchtweghyperreaktiviteit met direkte stimuli is niet eenduidig geassocieerd met een specifieke celparameter als maat van ontstekingsaktiviteit in de luchtwegen.

Een inhalatie-provokatietest met adenosine 5'-monophosphaat (AMP) is een waardevolle en veelbelovende parameter in het laboratoriumonderzoek voor het *in vivo* vervolgen van inflammatoire aktiviteit in de luchtwegen en het evalueren van het effekt van anti-inflammatoire medikamenteuze therapie in astma en COPD.

Roken is niet de belangrijkste faktor voor het ontstaan van longemfyseem.

Voor een adekwate bepaling van het totale aantal eosinofielen in het bloed dienen eosinofielen in een telkamer te worden geteld en niet uit het celdifferentiatie percentage van een bloeduitstrijkje te worden berekend.

Aktivatie van de zuurstofradikaalproduktie door bronchoalveolaire fagocyten speelt geen belangrijke rol bij het ontstaan van weefselbeschadiging tijdens de akute uitstoting van longtransplantaten bij de rat.
Am Rev Respir Dis 1992;145:1155-1159

Gezien de *in vitro* remming van de neutrofiele chemotaxis door kippesoep moet de anti-inflammatoire waarde van dit traditionele (genees-)middel niet worden onderschat.
Am Rev Respir Dis 1993;147:472.

Het uitvoeren van nachtelijk onderzoek versterkt bij de onderzoeker de overtuiging dat fysiologische chronobiologische ritmes dienen te worden gerespekteerd.

Vliegtuigen maken gebruik van hogere luchtwegen.

Promotiecommissie: Prof P.J. Barnes, MA, DM, DSc, FRCP
Prof. Dr. J.W.J. Lammers
Prof. Dr. J. Zaagsma

Ontwerp omslag: Henk van der Eerden

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INDIRECT AIRWAYS RESPONSIVENESS AND CELL ACTIVATION AS INFLAMMATORY PARAMETERS IN NOCTURNAL ASTHMA

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Promotores:	Prof. Dr. D.S. Postma
	Prof. Dr. G.H. Koëter
Referent:	Dr. H.F. Kauffman

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

In this thesis studies are presented with respect to the contribution of inflammatory processes in the occurrence of nocturnal dyspnea in asthma. Asthma is in this thesis considered as a syndrome with onset at young age, and is characterized by episodic (increases in) wheezing and dyspnea as a consequence of airflow limitation, the presence of airways hyperresponsiveness and atopy. Nocturnal dyspnea is a common feature in asthma and frequently disrupts sleep around 4:00 AM as a consequence of narrowing of the airway diameter. It has been recognized that the airway diameter shows a physiological circadian (= 24-hours) swing. In asthmatic patients with nocturnal dyspnea larger circadian swings in airway diameter occur than in healthy persons or asthmatic patients without nocturnal dyspnea (1). This phenomenon is generally called 'nocturnal asthma'. It must be recognized, however, that this feature is not exclusively observed in asthmatics, but may also occur in nonatopic subjects with chronic obstructive airflow limitation (2).

Since centuries it has been recognized that patients with asthma may suffer from severe attacks of dyspnea at night (3) and despite improvement of therapy in the last decades, nocturnal dyspnea is still common (4, 5), contributing to both increased asthma mortality at night and impaired daytime cognitive performance (6, 7). Therefore, it goes without saying that the origin of nocturnal asthma has been subject of great interest in asthma research during decades. More insight into the pathogenetic mechanisms underlying increased nocturnal airways obstruction offers the possibility to develop improved therapeutic approaches in order to prevent the annoying symptoms. In the following we will discuss the relevance of investigating inflammatory processes in the occurrence of nocturnal asthma and the parameters which have been applied in our studies.

Specific inflammatory processes have been recognized as a characteristic feature in the airways of asthmatic subjects (8). The knowledge of these inflammatory processes has largely extended over the last few years with the application of flexible bronchoscopy in asthma research and advances in immunopathological techniques, allowing to investigate the morphology, state of activation and mediator release of airway cells in assocation with the clinical manifestation of asthma. Besides, specific inflammatory changes have been reported to enhance the severity of airways hyper-responsiveness, one of the most prominent characteristic features of asthmatic individuals. Airways hyperresponsiveness contains the enhanced ability of the airways

to narrow on exposure of a small quantity of a wide variety of non-specific stimuli that do not provoke such a reaction in normal subjects (9). In the laboratory, the advantage of measurement of airways hyperresponsiveness is that it is a conventional technique that can easily be repeated in a standardized way. Up to now there are a few studies showing that airways responsiveness to histamine and methacholine are increased at night (10-12). This appears not only to happen when asthmatic patients experience a fall in FEV_1 at night, but also in asthmatic subjects who have a stable circadian rhythm in airway diameter (11, 12). Increased airways hyperresponsiveness at night may therefore be regarded as the first signal of a potential increased susceptibility to develop nocturnal airways narrowing. Airways narrowing, on its turn, is determined by both the thickness of the airway wall and the smooth muscle tone. Airway wall thickness may increase at night as a consequence of oedema, cell infiltration and other pathological changes due to local circadian inflammatory processes, whereas changes in the tone of the airways smooth muscle cells may be controlled by circadian variations in inhibitory adrenergic and nonadrenergic and excitatory cholinergic and noncholinergic stimuli of the autonomic nerve system. Many factors, proposed to explain the development of nocturnal airways narrowing (13), may contribute to the supposed increased inflammatory processes at night. The nocturnal fall in endogenous cortisol and adrenaline levels may intensify inflammatory responses. Besides, allergen exposure may result in recruitment and activation of inflammatory cells in the airways (14). Evidence that inflammatory activation is involved in the occurrence of nocturnal asthma also stems from findings in a previous study that adequate anti-inflammatory treatment with inhaled corticosteroids reduced the circadian variation in airways obstruction and hyperresponsiveness (15).

In order to get more insight in inflammatory processes contributing to nocturnal asthma, it was investigated whether specific inflammatory changes could be detected in atopic asthmatic subjects with increased nocturnal airways obstruction, differentiating them from atopic asthmatic subjects without increased nocturnal airways obstruction. Evidence for specific inflammatory changes was assessed in two different ways. Indirect information was obtained by studies on day-night variation of airways hyperresponsiveness to 'direct' and 'indirect' stimuli. More direct information was obtained by investigation of the numbers and activation of inflammatory cells, obtained by bronchoalveolar lavage, bronchial biopsy sampling and from peripheral blood.

Airways hyperresponsiveness can be assessed by a variety of directly and

indirectly acting stimuli. Methacholine is the most commonly used provocative stimulus, mainly causing smooth muscle contraction via direct action on the muscarinic receptors of these cells. Although an association has frequently been reported between the extent of inflammation in the airways and the degree of airways hyper-responsiveness to methacholine in asthma, it is not a stimulus that can be used as a parameter of specific inflammatory processes in the airways. In this respect, more information may be expected using stimuli that indirectly cause airways narrowing, among which adenosine 5'-monophophate (AMP) and propranolol, which act primarily on other - inflammatory or neuronal - cells, evoking processes that secondarily cause smooth muscle contraction (16).

Inhaled AMP is rapidly dephosphorylated to adenosine, which probably acts by stimulation of purinoceptors on cell surfaces (17). *In vitro* studies have shown that adenosine produced only a very weak contraction of human bronchiolar segments (18), indicating that a 'direct' effect on smooth muscle cells is unlikely. Although is it not known which cells exactly are involved in AMP-induced airways constriction, evidence exists that mast cell release of bronchoconstrictive mediators is induced (19), whereas neural reflex mechanisms may be involved as well (20, 21).

Propranolol acts by blockade of ß-adrenergic receptors, which are present on many different cell types located in the airway walls (22). Evidence for an indirect action on the cholinergic neurotransmission is supported by the protective effect of oxitropium bromide and atropine on propranolol-induced airways obstruction (23, 24). Besides, involvement of the nonadrenergic noncholinergic neurotransmission is supported by the finding that inhalation of vasointestinal peptide decreased propranolol airways responsiveness (25). Finally, pretreatment with cromoglycate protected against propranolol-induced airways obstruction, suggesting involvement of mast cell release or axon reflex mechanisms (26).

Although up to now the value of indirectly acting stimuli is limited, because the exact mode of action of the current indirectly acting stimuli is often unknown or thought to act via more than one pathway, assessment of indirect airways responsiveness may nevertheless provide information of inflammatory processes in the airways and is a field of interest that needs to be elucidated further. In this respect, we also evaluated the surplus value of AMP in two additional studies, not related with nocturnal asthma. The airways hyperresponsiveness to AMP was compared with that to methacholine in studies on smoking and nonsmoking subjects with asthma and

chronic obstructive pulmonary disease. These studies are included in the last part of this thesis.

1.2. Aims of studies

In this thesis questions were addressed with respect to the following subjects:

1. Which associations have been reported in literature between methacholine or histamine-induced airways hyperresponsiveness and the occurrence of specific inflammatory processes in the airways of asthmatic subjects? *Chapter 2. Inflammation and nocturnal asthma*
2. Is the occurrence of increased nocturnal airways constriction in atopic asthmatic subjects associated with an overall increase in the degree of airways responsiveness at daytime or by an increase in the day-night variation of airways responsiveness? Furthermore, is the increase in nocturnal airways constriction more associated with (day-night changes in) airways responsiveness to indirectly (AMP, propranolol) than to directly (methacholine) acting stimuli? *Chapter 3.*
3. What is the effect of propranolol inhalation tests on the diurnal increase in FEV_1 and propranolol airways responsiveness in atopic asthmatic subjects? *Chapter 4.*
4. Do day-night changes in inflammatory cell parameters in BAL-fluid and peripheral blood occur in atopic asthmatic subjects with and without increased nocturnal airways obstruction and in nonatopic healthy subjects? Are these changes associated with the occurrence of increased nocturnal airways obstruction in the asthmatic subjects? *Chapters 5 and 6.*
5. Does an influx of inflammatory cells in the bronchial submucosa occur at night in asthmatic and healthy subjects? *Chapter 7.*
6. Do results from studies on nocturnal asthma indicate that inflammatory changes at night underlie the occurrence of nocturnal asthma? *Chapter 8. Inflammation and AMP airways hyperresponsiveness*
7. Do subjects with COPD respond with airways narrowing upon stimulation with AMP to a similar degree as compared with subjects with asthma? What is the effect of smoking on the airways responsiveness to AMP in COPD and asthma? What is the surplus value in assessing AMP airways responsiveness above assessment of methacholine airways responsiveness? *Chapters 9 and 10.*

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Chapter 2

INFLAMMATION AND AIRWAYS HYPERRESPONSIVENESS IN ASTHMA

Ytske Oosterhoff, Dirkje S. Postma, Gerard H. Koëter

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Summary

Since the introduction of flexible bronchoscopy and advances in immunopathological techniques more knowledge of inflammatory processes in asthma has been gained. Inflammatory changes in the airways are already present in mild stable asthmatics, whereas local infiltration or activation of specific cell types is induced by exogenous modulation, *eg.* by allergen challenge and modulation during the night in nocturnal asthmatics. These inflammatory changes contribute to the clinical manifestation of airways hyperresponsiveness, which can be assessed by inhalation provocation tests with 'direct' (methacholine, histamine) or 'indirect' (adenosine 5'-monophosphate: AMP) stimuli. As yet, no provocative stimulus exists that can be used as a parameter of specific inflammatory changes in the airways. Relationships with methacholine or histamine-induced airways hyper-responsiveness have been found for various inflammatory parameters, but not consistently. Therefore, measurement of airways hyperresponsiveness by 'indirect' stimuli may better reflect specific inflammatory changes. Results from studies in our laboratory indicate that AMP is a more sensitive parameter than methacholine for measuring specific inflammation following the early asthmatic reaction after allergen challenge and in nocturnal asthma. Assessment of 'indirect' airways responsiveness, therefore, needs further investigation on its usefulness in clinical practice.

Introduction

As a result of the introduction of flexible bronchoscopy and advances in immunopathological techniques, evidence has become available that specific inflammatory processes in the airways underlie the pathogenesis of asthma (1). Different cells infiltrate in the intra-epithelial and submucosal layer of the bronchial walls, interacting with each other by release of cytokines and leading to pathological changes that contribute to airways hyperresponsiveness, one of the most prominent functional characteristics of asthma (2).

Airways hyperresponsiveness describes the enhanced ability of the airways to narrow on exposure of a small quantity of a wide variety of non-specific stimuli that do not provoke such a reaction in normal subjects (3). The presence of airways hyperresponsiveness can potentially lead to acute changes in the severity of airways narrowing, which on its turn is determined by both the thickness of the airway wall and the smooth muscle tone. Airway wall thickness may increase as a consequence of

oedema, cell infiltration and other pathological changes due to local (chronic) inflammatory processes (4), whereas changes in the tone of the smooth muscle cells are controlled by inhibitory adrenergic and nonadrenergic and excitatory cholinergic and noncholinergic stimuli of the autonomic nerve system (5).

The severity of airways hyperresponsiveness is modulated by several exogenous and endogenous factors. This modulation changes the airway diameter, which as a consequence of geometrical and anatomical factors, partly determines the degree of airways responsiveness as well (6,7). Allergen challenge (8) and occupational agents (9) are known to increase airways responsiveness up to several weeks, whereas exposure to tobacco smoke (10,11) and viral respiratory infections (12) may contribute to a temporarily increased airways responsiveness as well. Furthermore, airways responsiveness shows a circadian rhythm, being best in daytime and worst at night (13). Although the mechanism underlying these observations is not exactly understood, it is recognized that inflammatory processes are involved.

In clinical practice, provocation tests are usually performed with histamine or methacholine. These stimuli induce airways constriction mainly by 'direct' interaction with receptors on smooth muscle cells. Other stimuli, like adenosine 5'-monophophate (AMP), are thought to act by 'indirect' mechanisms through interaction with receptors on other cells (inflammatory or neuronal cells), evoking processes secundarily leading to smooth muscle contraction, eg. by release of inflammatory mediators and/ or stimulation of sensory nerve receptors, leading to local or central mediated nervous reflexes (14). Usually, 'direct' airways hyperresponsiveness is investigated in its relationship to inflammatory parameters. However, a relationship with airways hyperresponsiveness has been found for various inflammatory parameters, whereas these relationships are not consistently found in different studies.
Therefore, measurement of airways hyperresponsiveness by 'indirect' stimuli may better reflect specific inflammatory changes and may be preferred.

In the present paper, the role of inflammation in the clinical expression of airways hyperresponsiveness and its relationship with measurement of 'direct' and 'indirect' airways hyperresponsiveness will be addressed.

Inflammatory changes in the asthmatic airways

Evidence for airways pathology of asthmatics was already present as early as 1883 when respiratory epithelial cells in the sputum of asthmatic patients were found (15).

Postmortem studies of fatal asthma have shown mucous plugs in the airways containing inflammatory exudate and mucus with desquamated surface epithelial cells, lymphocytes and eosinophils (16), desquamation of the bronchial epithelium, goblet cell hyperplasia and (seemingly) thickening of the basement membrane. The latter was later recognized as deposition of collagen and other connective tissue products below the basement membrane (17).

More specific knowledge has become available since the introduction of flexible bronchoscopy in asthma research, allowing collection of cells and other constituents from the epithelial lining fluid by bronchoalveolar lavage (BAL) and small biopsy specimens of the bronchial mucosa (1). Thus, it has become evident that inflammatory changes in the airways are already present in mild stable asthmatics, whereas local infiltration or activation of specific cell types is induced by exogenous modulation, eg. by allergen challenge (18-21), and modulation during night in nocturnal asthmatics (22).

Eosinophil infiltration in the airways is characteristic in the inflammatory process in asthma. They can be recruited and activated in the airways by chemotactic cytokines such as platelet activating factor (PAF), interleukin-5 (IL-5), and granulocyte-macrophage colony stimulating factor (GM-CSF), released by activated T-lymphocytes, mast cells or macrophages (23,24). Increased eosinophil numbers have consistently been demonstrated in BAL (25,26) and biopsy specimens (27) of stable atopic and non-atopic asthmatics. The eosinophils are activated, as based on findings of increased numbers of EG-2-positive staining cells in biopsy specimens (28,29) and increased eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil derived neurotoxin (EDN) concentrations in the BAL fluid of (symptomatic) asthmatics (30-33). The release products of the eosinophil cause damage to the epithelium, which is reflected by increased epithelial shedding in biopsies (34) and epithelial cell numbers in BAL (26,27,32) of asthmatics. In association with the late allergic reaction (LAR), an increase in eosinophil numbers and activation products is observed in BAL and biopsy specimens 3 - 24 hours after allergen provocation (18-21). Also in nocturnal asthma, the eosinophil is found in increased numbers in BAL at night (22). Moreover, evidence for eosinophil activation can be concluded from increased blood ECP levels and number of hypodense eosinophils at night in nocturnal asthmatics (35,36).

Recent studies have shown activation of T-lymphocytes in the airways of stable asthmatics. The expression of the cell surface activation markers CD25, HLA-DR and VLA1 is increased on BAL lymphocytes and in biopsy specimens (28,26,37) from

stable asthmatics, whereas evidence for specific activation of the T_{H2} lymphocyte is supported by findings of increased mRNA for IL-2, IL-3, IL-4, IL-5 and GM-CSF signals on BAL lymphocytes (38), biopsy specimens (39) and in BAL fluid (26) of stable asthmatics. Also 24 hours after allergen challenge the GM-CSF expression on BAL T lymphocytes is increased (40).

Mast cells in the airways of stable asthmatics appear to be activated, as electron microscopy studies in biopsy material reveal degranulated mast cells (29, 41). This is supported by increased BAL levels of histamine, tryptase and PGD_2 (20, 42, 43). After allergen challenge, activation of mast cells plays a pivotal role in the pathogenesis of the immediate asthmatic reaction through their release of histamine, PGD_2 and LTC_4. It is, however, possible that they also have a role in initiating and attracting other cells to generate the LAR, eg. by release of PAF. Recently it was reported that airway mast cells contain preformed TNFα, IL-4, IL-5 and IL-6 (44), suggesting involvement of mast cells in eosinophil recruitment and IgE-synthesis as well. The observed increase in urinary N^{τ}- methylhistamine excretion in children with nocturnal asthma (45) and plasma histamine levels in adults (46) suggest a role for mast cells in the pathogenesis of nocturnal asthma.

The contribution of alveolar macrophages (AM) in the pathogenesis of asthma is less clear. AM are abundantly present in the airways. An influx of monocyte-like cells is found in BAL and biopsy specimens of stable extrinsic (47, 48) and intrinsic (26) asthmatics and after allergen challenge (49). Activation of AM has been reported, as concluded from their enhanced chemiluminescence in stable asthmatics (50) and after allergen challenge (51). AM are capable of generating over a hundred products (52). When challenged with antigen, they secrete chemotactic factors for eosinophils and neutrophils (53). AM from asthmatics release PAF-acether on challenge with specific antigen (54), whereas those from nonallergic controls or treated asthmatic individuals do not. They are also capable of releasing GM-SCF, and may therefore play a role in attraction of the eosinophils to the lung.

In conclusion, evidence is growing from studies in stable asthmatics, as well as studies after allergen challenge and on nocturnal asthma, that a T-cell mediated specific process underlies the pathogenesis of asthma, in which eosinophil recruitment plays an important role. Many other cell types - mast cells, macrophages - may be involved as well, evoking inflammatory processes that together determine the pathological changes underlying the clinical manifestation of asthma.

Relationship between the degree of airways hyperresponsiveness and inflammatory changes in the airways

'Direct' airways hyperresponsiveness

Indirect evidence for the contribution of inflammation to airways hyperresponsiveness is drawn from studies showing that treatment with anti-inflammatory drugs attenuates the airways hyperresponsiveness. Long-term inhalation with corticosteroids resulted in a continuing reduction in airways hyperresponsiveness in asthmatics (55, 56), whereas cessation of steroid inhalation showed rapid deterioration of the airways hyperresponsiveness (57). Despite treatment of asthmatics with inhaled corticosteroids during 10 years, airways hyperresponsiveness was neverteheless still present, although the number of inflammatory cells was significantly decreased in biopsy specimens (58).

Although inflammatory processes are involved, it is not known how these processes translate to airways hyperresponsiveness. Several studies show a relationship between different parameters of inflammation and the degree of airways hyperresponsiveness as measured with methacholine or histamine (Table 1). However, relationships between specific inflammatory parameters and the degree of airways hyperresponsiveness have not consistently been found, whereas the strength of their relationship suggests that airways hyperresponsiveness is not solely determined by inflammation. It may well be that methacholine or histamine are not the optimal tools to detect airways inflammation, as they supposedly act directly on the airways smooth muscle cells. Therefore, other, 'indirect' stimuli have been tried as well, one of them being AMP.

'Indirect' airways hyperresponsiveness

AMP

Mechanism of action. After inhalation AMP is rapidly metabolised to adenosine. A direct action of adenosine on smooth muscle cells is thought unlikely, as adenosine induces only a weak contraction of human bronchiolar segments *in vitro* (65). Mast cell release of histamine is a major pathway of adenosine induced airways constriction, as pretreatment with H_1-antihistamines reduced the response by up to 80% (66). Besides, prostaglandin release is involved (67). *In vitro* experiments have shown that mast cell release is induced via stimulation of A_2-purinoceptors (68). Although A_2-purinoceptors are present on many other cells types, eg. eosinophils, neutrophils and macrophages, no evidence exists that these cells are involved in the adenosine-induced airways

Table 1. Relationships between 'direct' airways hyperresponsiveness and inflammatory parameters in the airways of (stable) asthmatics

Parameter	Biopsy/BAL	His/Meth provocation	Correlation*	Reference	
Number of	biopsy	M	-	*Ollerenshaw*	(59)
inflammatory cells	biopsy	M	-	*Bradley*	(28)
	biopsy	M	+	*Foresi*	(60)
Epithelial loss	biopsy	M	+	*Jeffery*	(34)
Epithelial cells	BAL	M	+	*Foresi*	(60)
	BAL	H	+	*Beasley*	(27)
	BAL	M	-	*Kelly*	(50)
	BAL	M	+	*Wardlaw*	(30)
Eosinophils	biopsy	M	+	*Bradley*	(28)
	biopsy	M	-	*Djukanovic*	(29)
	biopsy	H	+	*Laitinen*	(61)
	BAL	H	+	*Jarjour*	(62)
	BAL	M	+	*Walker*	(37)
	BAL	M	-	*Kelly*	(50)
	BAL	M	+	*Kirby*	(25)
	BAL	M	+	*Wardlaw*	(30)
MBP	BAL	M	-	*Kirby*	(25)
	BAL	M	+	*Wardlaw*	(30)
Mast cells	biop	M	-	*Bradley*	(28)
	biop	M	-	*Djukanovic*	(29)
	BAL	M	+	*Kirby*	(25)
	BAL	M	+	*Wardlaw*	(30)
Tryptase	BAL	H	-	*Jarjour*	(62)
Histamine	BAL	H	+	*Jarjour*	(62)
T-lymphocytes	biopsy	M	-	*Bradley*	(28)
	biopsy	M	-	*Poulter*	(47)
	BAL	M	-	*Walker*	(37)
	BAL	M	+	*Kelly*	(63)
Activated T-lymphocytes	BAL	M	+	*Walker*	(37)
Chemiluminescence of macrophages	BAL	M	+	*Kelly*	(50)
Neutrophils	BAL	M	+	*Kelly*	(50)
Albumin, ceruloplasmin, α_2-macroglobulin	BAL	H	+	*Van de Graaf*	(64)

* + = present, - = absent; His (H) = histamine, Meth (M) = methacholine

constriction. In contrast, adenosine inhibited the *in vitro* superoxide anion production by eosinophils and neutrophils (69, 70), which rather suggests a protecting role of adenosine.

In addition to mast cell release, it has been suggested that neural reflex mechanisms are involved in adenosine-induced bronchoconstriction. This is supported by findings that muscarinic antagonists and lidocaine opposed the adenosine-induced bronchoconstriction (71, 72).

Relationship with inflammatory parameters. Relationships between parameters of inflammation obtained from BAL and biopsy studies and the degree of airways hyperresponsiveness as measured by provocation with adenosine or AMP have as yet scarcely been investigated. In rats, bronchoconstriction induced by intravenous injection of adenosine (and neurokinin A) is associated with increased histamine levels in the BAL-fluid (71), suggesting enhancement of mast cell release. This is supported by a study in which asthmatics who were hyperresponsive to adenosine showed a significant correlation between the degree of reduction in FEV_1 and an increased serum neutrophil chemotactic activity, as a marker of mast cell degranulation (73).

Recently, in our laboratory, studies on AMP and methacholine airways responsiveness have been performed in allergic asthmatics after allergen provocation and in a study on nocturnal asthma.

Aalbers and coworkers (74) studied the airways hyperresponsiveness to methacholine and AMP in nine allergic patients (dual responders) 3 and 24 hours after allergen challenge (Figure 1). PC_{20} AMP was significantly decreased 3 hours post-

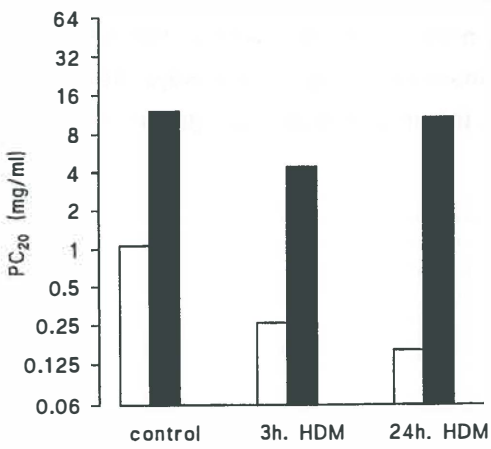

Figure 1. PC_{20} methacholine (open bars) and PC_{20} AMP (closed bars) 3 h after control solution (*control*), and 3 and 24 after house dust mite (*HDM*) challenge in 9 allergic patients.

challenge, but not 24 hours after challenge. In contrast, PC_{20} methacholine was significantly decreased at both time points. These findings suggest that AMP challenge may reflect specific inflammatory changes following the early asthmatic reaction.

In our study on nocturnal asthma (Chapter 3), the airways hyperresponsiveness to methacholine and AMP was assessed at 04.00 and 16.00 hours in 16 allergic asthmatics, divided in a group with and without increased nocturnal airway obstruction, based on an increased circadian peak flow variation of $\geq$ 15%. In the group with increased airway obstruction at night, the nocturnal geometric mean PC_{20} AMP decreased more than the PC_{20} methacholine value (2.3 and 0.9 doubling dilutions, respectively; $p < 0.05$), whereas in the group without increased nocturnal airway obstruction the AMP and methacholine PC_{20} values during day and night were equal. The circadian change in airways responsiveness to AMP correlated significantly with the circadian PEFR- variation ($r = 0.81$, $p < 0.001$), in contrast to methacholine ($r = 0.14$, no significant difference). These findings suggest that an increased susceptibility to mediator release by mast cells or neural reflex mechanisms is involved in the occurrence of nocturnal asthma.

Conclusions

Specific inflammatory processes in the asthmatic airways lead to pathologic changes that contribute to airways hyperresponsiveness. As yet, no provocative stimulus exists that can be used as a parameter of specific inflammatory changes in the airways. Results from studies in our laboratory indicate that AMP provocation is a more sensitive parameter than methacholine for measuring specific inflammatory, probably mast cell related changes following the early asthmatic reaction after allergen exposure and during night in nocturnal asthma. 'Indirect' hyperresponsiveness may better reflect inflammatory changes in the airways. Assessment of 'indirect' hyperresponsiveness needs, therefore, further investigation on its usefulness in clinical practice.

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Chapter 3

CIRCADIAN VARIATION IN AIRWAYS RESPONSIVENESS TO METHACHOLINE, PROPRANOLOL, AND AMP IN ATOPIC ASTHMATIC SUBJECTS

Ytske Oosterhoff, Gerard H. Koëter, Jan G.R. de Monchy, Dirkje S. Postma

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Abstract

Increased airways hyperresponsiveness is thought to be one of the phenomena underlying nocturnal airways obstruction in asthma. To investigate the mechanisms that influence and modulate this phenomenon, we compared circadian variations in airways responsiveness to AMP and propranolol with the circadian variation in airways responsiveness to methacholine. Inhalation provocation tests were performed at 16.00 and 04.00 h in 16 nonsmoking atopic asthmatic subjects (18 to 42 yr of age), prospectively assigned to Group 1 (mean circadian peak expiratory flow rate [PEFR] variation $\geq$ 15%) and Group 2 (< 15%). The circadian change in airways responsiveness to AMP, in contrast to methacholine, was significantly related to the circadian PEFR-variation of the 16 subjects ($r = 0.81$, $p < 0.001$). In Group 1 ($n = 7$) geometric mean PC_{20} AMP decreased more than PC_{20} methacholine during the night (2.3 and 0.9 doubling concentrations respectively, $p < 0.05$), whereas no difference in baseline FEV_1 was found at the same time points during the different study days. Geometric mean PC_{20} propranolol did not change during the night. Daytime PC_{20} propranolol and PC_{20} AMP, in contrast to PC_{20} methacholine, were significantly lower in Group 1 as compared with Group 2. Together, the results show a higher susceptibility to stimulation of 'indirect' airways responsiveness in the subjects with increased circadian PEFR amplitude. This suggests that mast cell activation rather than primary changes in smooth muscle cell contraction may play a role in the development of nocturnal airways obstruction.

Introduction

Studies on nocturnal asthma have demonstrated that airways hyperresponsiveness to histamine and methacholine is increased in asthmatic individuals during the night (1, 2). Airways hyperresponsiveness is thought to be one of the phenomena underlying nocturnal airways obstruction, which may lead to recurrent nocturnal dyspnea, life threatening nocturnal attacks or even death (3). Development of nocturnal airways obstruction has been suggested to result from an exaggeration of the normal circadian rhythm in airway caliber controlled by the autonomic nervous system. Both endogenous factors related to circadian chronobiological rhythms, such as circulating hormone levels (including epinephrine) and environmental factors, such as allergen exposure, are supposed to contribute to the increase in the severity of nocturnal airways responsiveness (4, 5). The mechanisms by which these factors operate are not fully

understood, but evidence is growing that inflammatory processes may be responsible for the increase in nocturnal airways responsiveness (6).

Airways responsiveness can be measured by different stimuli. In this respect, challenges with histamine and methacholine cause airways narrowing mainly by direct action on receptors of airways smooth muscles, whereas stimuli like AMP and propranolol are thought to act primarily on other cells, thus initiating processes which secondarily ('indirectly') lead to smooth muscle contraction (7). The exact mechanism leading to airways obstruction induced by AMP inhalation is as yet unclear. Inhaled AMP is rapidly dephosphorylated in the lung to adenosine, which probably acts by stimulation of purinoceptors on cell surfaces (8). Results from different investigations indicate that a direct effect of adenosine on smooth muscle cells is unlikely, whereas stimulation of mast cells or an action via neuronal reflexes in the lung are probably involved in the adenosine-induced airways constriction.

Propranolol inhalation induces airways constriction by blockade of beta-adrenergic receptors. Results from different studies have shown involvement of beta-receptor blockade on stimulation of the cholinergic pathway (9), on vasoactive intestinale peptide release (10) and on histamine release by mast cells (11).

If inflammatory processes are involved in the nocturnal increase in airways responsiveness, assessment of indirect rather than direct airways responsiveness might better reflect the *in vivo* situation. Moreover, such stimuli might provide more insight in the pathogenetic mechanisms leading to increased nocturnal airways obstruction. Therefore, we compared circadian variations in indirect airways responsiveness to AMP and propranolol with direct airways responsiveness to methacholine. This was assessed in atopic asthmatic subjects, who were prospectively assigned to a group with or without increased mean circadian peak expiratory flow rate (PEFR) amplitude.

Methods

Subjects

Sixteen atopic nonsmoking asthmatic individuals participated in the study. All subjects had a history of episodic dyspnea or wheezing consistent with the clinical diagnosis of asthma and had no concomitant diseases. None of the subjects had taken oral corticosteroids within 2 months before the study. Inhaled corticosteroids, if used, were stopped four wk before onset of the study, whereas cromoglycate and theophylline medication were discontinued two wk before. Selection criteria at enrollment in the

study were as follows: (*1*) age between 18 and 45 yr; (*2*) atopy: elevated specific IgE against house dust mite (RAST score $\geq$ 0.7 PRU/ml; Phadezym^{R} RAST Pharmacia, Uppsala, Sweden) or positive intra-cutaneous tests against house dust mite or two other of 11 tested common aeroallergens (Diephuis Laboratories, Groningen, the Netherlands); (*3*) FEV_1 > 1.50 l and > 60% predicted; post-bronchodilator FEV_1 (after salbutamol 200 μg using a spacehaler) $\geq$ 80% predicted; (*4*) airways hyper-responsiveness to histamine, defined as the provocative concentration of histamine that caused a 20% fall in FEV_1 (PC_{20}) of $\leq$ 32 mg/ml (30" inhalation, 2' interval; values in healthy individuals are > 32 mg/ ml).

The subjects were recruited from our outpatient clinic of the Department of Pulmonary Diseases and from advertisements in local newspapers. All subjects signed an informed consent agreement. The study was approved by the study center hospital ethics committee.

Study design

From 4 wk before the start of the study subjects were allowed to use inhaled broncho-dilators only (salbutamol or ipratropiumbromide). At 2 wk before the study, the subjects scored nocturnal asthma symptoms and recorded peak expiratory flow (PEF) values at home obtained with a Wright mini peak flow meter. Nocturnal asthma symptoms were recorded during 11 nights and scored as the number of nights in which the subjects awoke because of nocturnal dyspnea and/ or used inhaled bronchodilators.

In the second week PEF values were recorded during three days every 4 h during 24 h, i.e. at 08.00, 12.00, 16.00, 20.00, 24.00 and 04.00 h. The best of three PEFR maneuvers was recorded. The mean circadian PEFR-variation of the three days was defined as: (highest - lowest 24 h value)/ mean 24 h value. Subjects were prospectively assigned to *Group 1* if the mean circadian PEFR-variation was $\geq$ 15% and to *Group 2* when the mean circadian PEFR-variation was < 15%.

In the following ten days, PEFR measurements and inhalation provocation tests with methacholine, propranolol and AMP were performed on 3 different study days and in a fixed sequence to standardize possible interfering effects of one challenge on the others. Methacholine provocation tests were performed on Day 1, followed at least 2 days later by propranolol provocation tests (Day 2) and 1 wk later by AMP provocation tests (Day 3). The tests were performed at 16.00 h and the next morning at 04.00 h. Subjects waited in a waiting room during 15 minutes before testing at 16.00 h.

Subjects were admitted to hospital and awakened 10 min before the measurements at 4:00 A.M. All medication was withheld 12 h before the tests and during hospital admission. Baseline FEV_1 had to be reproducible within 10% on the 3 different study days.

Methacholine, propranolol and AMP inhalation tests

Initial spirometry was performed using a calibrated water-sealed spirometer according to standardized guidelines (12). FEV_1 was measured until three reproducible (< 5% difference) recordings were obtained. Highest values were used for baseline prechallenge values. Solutions of methacholine, propranolol and AMP were stored at 4° C and administered at room temperature. After inhalation of 0.9% sodium chloride solution, doubling concentrations of methacholinechloride (0.125 to 16 mg/ml in normal saline), propranolol (0.5 to 32 mg/ml in normal saline) or AMP (0.04 to 320 mg/ml in normal saline) were inhaled by a 2-min tidal breathing method adapted from Cockcroft and coworkers (13). Aerosols were delivered by a DeVilbiss 646 nebulizer (DeVilbiss Health Care Inc., Somerset, PA), connected to the central chamber of a newly developed inspiratory and expiratory valve box of a bronchial provocation apparatus (BPA-2). Solution output was 0.13 ml/min while the air pressure control was adjusted to 1 atm. The challenge was discontinued when FEV_1 had fallen by $\geq$ 20% of the prechallenge level. PC_{20} values were calculated by linear interpolation of the last two points of the log concentration response curve.

Data analysis

Values are presented as mean (SD). PC_{20} values were analyzed after base 2 logarithmic transformation, a change of 1 U in $\log_2 PC_{20}$ representing one doubling concentration. All analyses were performed with the SPSS/PC$^+$ V4.0 software package (SPSS Inc., Chicago, IL). P values $\leq$ 0.05 were considered statistically significant. After verifying the normal distribution of values, changes in FEV_1, PEFR and PC_{20} within subjects were analyzed by using Student's paired, two-tailed t-test. Changes in parameters between groups were analyzed by using Student t-test for unpaired observations. The number of positive skin prick tests and nocturnal asthma symptoms between groups were compared by using the Mann-Whitney U test. Correlations between variables were performed with the Pearson correlation test. Baseline FEV_1 values at the different study days were compared using analysis of variance.

Results

Subjects

Subject characteristics are listed in table 1. Seven subjects were included in Group 1 (mean circadian PEFR variation $\geq$ 15%), and 9 were placed in Group 2 (mean circadian PEFR variation < 15%). There was no significant difference in age, sex or number of positive intracutaneous skin tests between the two groups. In Patient 10, who refused skin tests, the atopy was confirmed by elevated levels of specific IgE against house dust mite.

FEV_1 % predicted at enrollment was significantly lower in Group 1 as compared with Group 2 (73.1 $\pm$ 9.1 and 93.1 $\pm$ 12.1 %, respectively; p = 0.002). Although

Table 1. Subject characteristics

Subject No.	Age (*yr*)	Gender	FEV_1 (*% pred*)	No. positive skin tests	Medication use[1]	Symptoms score NA[2]	PEFR variation [3]%
Group 1: mean circadian PEFR variation $\geq$ 15 %							
1	26	M	80	2	IC	0	17
2	28	M	64	1	IC + B	0	21
3	21	F	67	6	Cr + B	4	23
4	31	M	79	5	IC + B	7	26
5	39	F	86	7	IC + B	4	27
6	36	M	62	4	B	2	33
7	30	F	74	4	IC + A	3	45
Mean	30.1		73.1*	5.0		2.4	27.6
SD	6.0		9.1	2.4		2.5	9.1
Group 2: mean circadian PEFR variation < 15 %							
8	20	F	97	2	Cr + A + T	1	7
9	42	F	82	2	A	0	8
10	20	M	82	nd	IC + A	1	9
11	32	M	103	6	IC	0	9
12	28	F	108	6	IC + B	1	10
13	38	M	92	4	Cr + A	0	10
14	24	F	111	2	B	0	12
15	22	M	80	1	B	2	13
16	23	F	83	6	IC + B	0	13
Mean	27.7		93.6	4.0		0.6	10.0
SD	8.0		12.1	2.6		0.7	2.3

Definition of abbreviations: ND = not done; A = anticholinergic, B = $ß_2$ adrenergic, Cr = cromoglycate inhalation, IC = inhaled corticosteroids, T = oral theophylline. 1. Regular therapy until 4 wk before participation in the study; 2. Nocturnal asthma symptoms recorded during 11 nights, scored as the number of nights in which the subjects awoke because of nocturnal dyspnea and/ or used an inhaled bronchodilator during night; 3. expressed as: (highest - lowest 24 h PEFR value)/mean 24 h PEFR value. * p = 0.002 compared with Group 2.

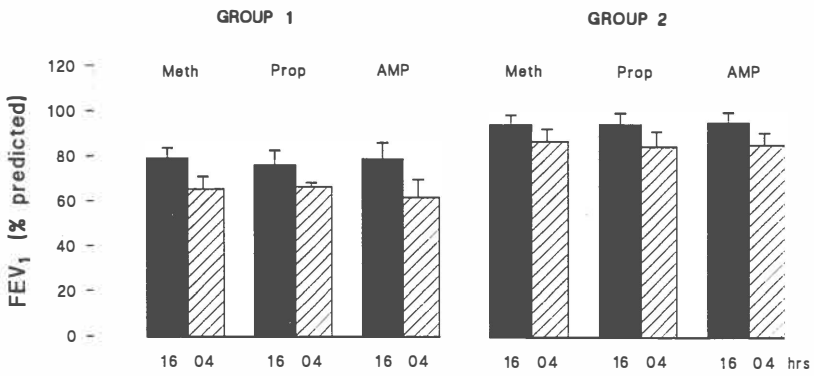


Figure 1. Mean (± SEM) baseline values of FEV_1 % predicted in Group 1: mean circadian PEFR variation ≥ 15% (*left*) and Group 2: mean circadian PEFR variation < 15% (*right*) at the 3 different study days. For further details, see text. Hatched bars = 4.00 h; solid bars = 16.00 h.

subjects in Group 1 did not wake up on significantly more nights because of nocturnal dyspnea than in Group 2 ($p = 0.08$), nocturnal asthma symptoms tended to be scored higher in the individuals with the largest PEFR- variations.

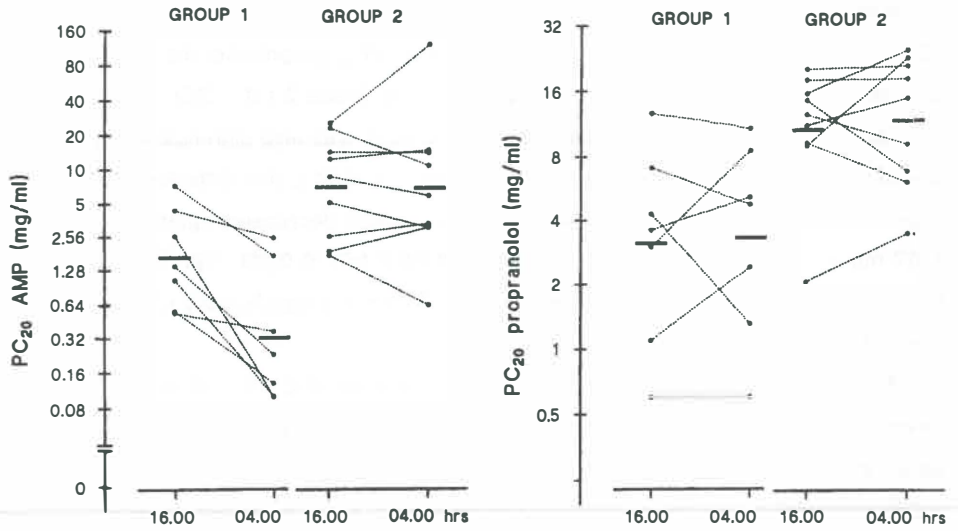
Baseline FEV_1

Mean baseline FEV_1 % predicted values are shown in figure 1. Within both groups there was no significant difference between the mean baseline FEV_1 % predicted values on the 3 different study days at the same time points ($p > 0.05$). In both groups FEV_1 fell significantly during night as compared with 16.00 h values. The mean fall in Group 1 (from 78.0 to 64.3%) was larger than in Group 2 (from 94.5 to 85.2%).

Provocation tests

Individual and geometric mean PC_{20} methacholine, propranolol and AMP values at 16.00 and 04.00 h of both groups are shown in figure 2.

Geometric mean PC_{20} methacholine values at 16.00 h did not significantly differ between the two groups (figure 2A), although the value tended to be lower in Group 1 than in Group 2 (0.36 and 0.79 mg/ml respectively). In Group 1, the geometric mean PC_{20} value decreased significantly during the night (0.19 mg/ml) as compared with the 16.00 h value; this decrease was 0.9 doubling concentration (DC) ($p = 0.038$). In



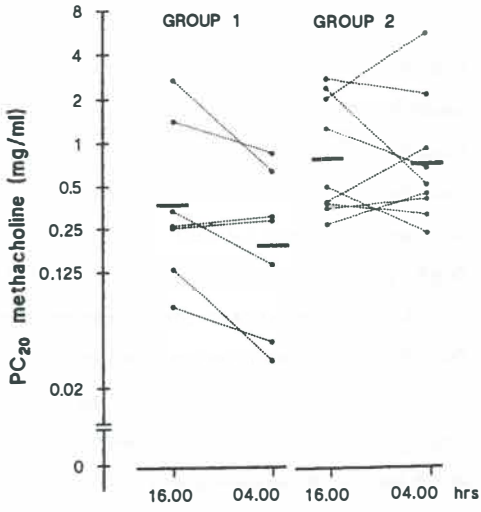


Figure 2. Airways responsiveness to methacholine, propranolol and AMP at 16.00 and 04.00 h in Group 1: mean circadian PEFR variation $\geq$ 15% (*left*) and Group 2: mean circadian PEFR variation $\leq$ 15% (*right*). Individual values are represented by dots. Mean values, expressed as geometric mean, are represented by horizontal bars. A change of 1 U in $\log_2 PC_{20}$ equals a change of one doubling concentration.

Group 2 the geometric mean PC_{20} value did not significantly decrease during the night (0.71 mg/ml;0.2 DC).

The geometric mean PC_{20} propranolol value at 16.00 h in Group 1 was significantly lower than the value in Group 2 (3.1 and 11.0 mg/ml respectively, $p = 0.02$; figure 2B). This difference was 1.8 DC. Geometric mean PC_{20} propranolol did not significantly change from 16.00 to 04.00 h in either Group 1 or Group 2 (-0.1 DC).

The geometric mean PC_{20} AMP value at 16.00 h was also significantly lower in Group 1 than in Group 2 (1.67 and 7.11 mg/ml, $p = 0.01$); this difference was 2.1 DC (figure 2C). In Group 1 the geometric mean PC_{20} value decreased significantly from 1.67 mg/ml at 16.00 hrs to 0.33 mg/ml at 04.00 h ($p = 0.005$), representing 2.3 DC. In Group 2, however, geometric mean PC_{20} AMP did not significantly change during the night (6.96 mg/ml, 0.03 DC).

FEV_1 fell only slightly from baseline after inhalation of 0.9% sodium chloride solution in both groups, results being comparable at 16.00 h and 04.00 h on the 3 study days. In Group 1 the mean fall in FEV_1 was 5.3 $\pm$ 3.6% at 16.00 h and 4.6 $\pm$ 4.0% at 04.00 h, in Group 2: 4.1 $\pm$ 2.4% and 3.0 $\pm$ 1.7% respectively.

Correlation of (circadian changes in) FEV_1 with circadian PEFR - variation

Mean baseline FEV_1 % predicted values from the 3 study days at 16.00 h were significantly correlated to the circadian PEFR variation ($r = -0.50$, $p < 0.05$). No significant correlations were found between the mean circadian change (16.00 h - 04.00 h) in FEV_1 and the circadian PEFR variation ($r = 0.26$, NS).

Correlation of circadian changes in airways responsiveness with circadian PEFR variation

$Log_2 PC_{20}$ values of methacholine, propranolol and AMP at 16.00 h were all significantly correlated to the circadian PEFR variation, but for propranolol and AMP the correlation was stronger than for methacholine (AMP: $r = -0.64$, $p = 0.007$; propranolol: $r = -0.65$, $p = 0.006$; methacholine: $r = -0.53$, $p = 0.03$).

A highly significant correlation between the change of log_2 PC_{20} AMP (16.00 h - 04.00 h) and the circadian PEFR variation was found ($r = 0.81$, $p < 0.0001$) for values of all subjects (figure 3). No significant correlations were found between the change of log_2 PC_{20} methacholine or propranolol and the circadian PEFR- variation ($r = 0.14$ and 0.18, respectively).

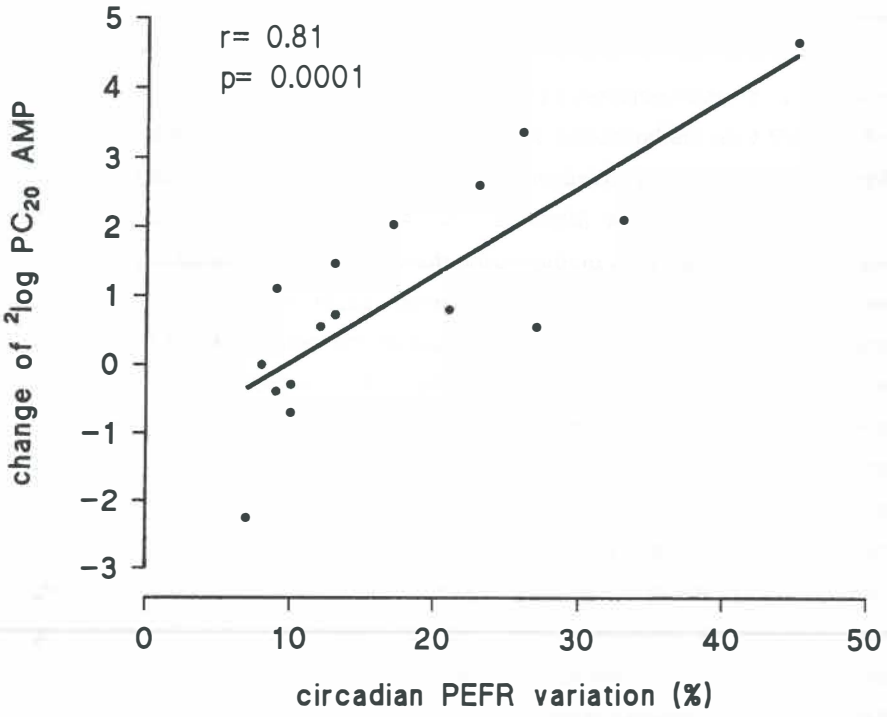


Figure 3. Correlation of change in $\log_2 PC_{20}$ AMP (16.00 - 04.00 h) with the mean circadian PEFR variation.

PEFR measurements at home and in the hospital

In Group 1 the mean PEFR % predicted value at 04.00 h recorded in hospital during 3 nights was significantly higher as compared to the values recorded during 3 nights at home ($p = 0.05$; table 2), whereas no changes were observed between the daytime values or the PEFR % predicted values in Group 2.

Table 2. Mean PEFR values (% predicted) recorded at home and in the hospital

	04.00 h		16.00 h	
	home	hospital	home	hospital
Group 1, circadian PEFR variation $\geq$ 15%	78 (10)	86 (16)*	99 (15)	96 (15)
Group 2, circadian PEFR variation < 15%	97 (10)	96 (8)	102 (11)	100 (9)

* p = 0.05 compared with value at home.

Discussion

This study demonstrates that the occurrence of an increased circadian variation in airway diameter is better reflected by circadian changes in airways responsiveness induced by AMP than methacholine. A highly significant correlation between the circadian PEFR-variation and circadian change in airways responsiveness to AMP was found, but not to methacholine. Moreover, PC_{20} AMP decreased 1.5 doubling concentrations more than PC_{20} methacholine during the night in the group with increased circadian PEFR amplitude. This difference could not be explained by differences in baseline FEV_1, as these were equal at the same points of time on the different study days. These findings suggest that indirect mechanisms are involved in the development of nocturnal airways obstruction, which are specific for AMP, as PC_{20} propranolol did not show circadian changes.

Although PC_{20} propranolol did not change during the night, we found that both the daytime PC_{20} propranolol and PC_{20} AMP values, in contrast to PC_{20} methacholine, were significantly lower in the group with increased circadian PEFR variation as compared to the group with a PEFR variation < 15%. This suggests that the occurrence of a large circadian variation in airway diameter is associated with an increased susceptibility to stimulation of indirect airways responsiveness.

As we performed PC_{20} measurements at 12-h intervals, development of tachyphylaxis might explain the unchanged values of propranolol. Although tachyphylaxis has so far not been described for propranolol, it has been reported for many other stimuli when repeated tests are performed (14, 15), but only within a 6 hour interval. This also indicates that tachyphylaxis can explain neither the unchanged PC_{20} methacholine nor the PC_{20} AMP values at night in Group 2.

In contrast to Martin and coworkers (16), who found a marked nocturnal fall in FEV_1 of more than 20% after saline inhalation in 8 of the 20 asthmatic subjects studied, we did not observe this phenomenon in any of our subjects. This may be explained by the fact that our subjects had only mild to moderate disease, with higher FEV_1 baseline values and less pronounced nocturnal falls in FEV_1.

Results from this study also show that daytime FEV_1% predicted values were significantly lower in the group with increased circadian PEFR variation, suggesting that the occurrence of an increased circadian variation in airways diameter is associated with more severe daytime airways obstruction. This is supported by a weak, but statistically significant correlation between daytime FEV_1 and the circadian PEFR

variation. However, we found no correlation between the diurnal change in FEV_1 and circadian PEFR variation. This can be explained by the fact that the increase in nocturnal airways obstruction during the stay in the hospital appeared to be smaller than at home: in the group with increased circadian PEFR variation the mean PEFR value recorded at 04.00 h in hospital was significantly lower than the 04.00 h values at home. The subjects spent the daytime in the same environment when they performed PEFR measurements. We therefore hypothesize that exposure of environmental factors at home during the night has an immediate effect on the increase in nocturnal airways obstruction. This suggests that allergen avoidance in the bedroom may lead to a reduction of nocturnal airways obstruction.

Based on a mean circadian PEFR variation of $\geq$ or $<$ 15%, we prospectively divided the asthmatic subjects in two groups. The division is, however, artificial, as it is known that the PEFR variability has a continuous distribution in the general population and in asthma (17, 18). By establishing the cutoff point at 15% we cannot exclude that subjects assigned to the group with a circadian PEFR of $\geq$ 15% were falsely labeled as having an "exaggeration of normal circadian rhythm". It is, therefore, the more remarkable that we found such big differences in airways responsiveness between both groups with the cutoff point at 15%.

The pathogenetic mechanism underlying the circadian change in AMP induced airways responsiveness is not fully understood. It is as yet unknown exactly which cells are stimulated by AMP to initiate processes leading to smooth muscle contraction. Results from other studies have suggested that release of histamine and other preformed mediators by airways mast cells plays an important role in the AMP induced airways obstruction (19, 20). Increased urinary levels of N^{τ}-methylhistamine (21) have been found during the night in allergic asthmatic children with increased nocturnal airways obstruction. This suggests that a reduced inhibition of histamine release, possibly in relation to the circadian variation of epinephrine serum concentrations, underlies the nocturnal fall in pulmonary function in asthma. If mast cell release is the main pathway by which AMP exerts its effect, the results of this study would be in agreement with these previous observations which indicate that the susceptibility of mast cells to mediator release is increased at night in asthmatic subjects with increased nocturnal airways obstruction. However, mast cell release may not be the only pathway underlying the development of increased nocturnal airways obstruction. Neuronal reflex mechanisms (22, 23) or other inflammatory mechanisms may play a

role as well.

In conclusion, our results show that the circadian change in airways responsiveness to AMP is significantly related to the circadian PEFR variation. Moreover, daytime PC_{20} AMP and PC_{20} propranolol, being both indirect stimuli of airways smooth muscle, were significantly lower in the group with increased circadian PEFR amplitude than in the group with a circadian PEFR amplitude < 15%. The higher susceptibility to stimulation of 'indirect' airways responsiveness suggests that mast cell activation rather than changes in smooth muscle cell contractiliy play a role in the development of nocturnal airways obstruction.

Acknowledgements

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Chapter 4

EFFECTS OF PROPRANOLOL INHALATION ON THE DIURNAL INCREASE IN FEV_1 AND ON PROPRANOLOL AIRWAYS RESPONSIVENESS IN ATOPIC ASTHMATICS

Ytske Oosterhoff, Gerard H. Koëter, Dirkje S. Postma

Submitted

Abstract

Propranolol inhalation provocation tests are used to measure indirect airways responsiveness in the investigation of asthma. In this study the effects of repeated propranolol inhalation provocation tests within the same day on normal diurnal variation in FEV_1 and subsequent propranolol airways responsiveness were investigated. Fifteen atopic asthmatic subjects were challenged with doubling concentrations of propranolol at 8 AM and 4 PM on the same study day and at 4 PM on a control day, to investigate changes related with normal diurnal variation.

Mean baseline FEV_1 on the study day at 4 PM was significantly lower as compared to the value on the control day at 4 PM (3.38 $\pm$ 0.23 l vs. 3.70 $\pm$ 0.24 l respectively; $p = 0.001$). No significant differences were found between geometric mean PC_{20} propranolol values measured on the study day (8 AM: 9.3 and 4 PM: 11.3 mg/ml) and on the control day at 4 PM (9.3 mg/ml).

Our results suggest that propranolol provocation at 8 AM has a long lasting effect on FEV_1, thereby opposing the normal diurnal increase in airway diameter, but has no effect on airways responsiveness to propranolol within a time interval of eight hours.

Introduction

Propranolol inhalation provocation tests are used to measure indirect airways responsiveness in the investigation of asthma (1). Assessment of indirect airways hyperresponsiveness is gaining interest as it may be a more optimal tool to reflect inflammatory processes in the airways of asthmatic subjects than measurement of direct airways hyperresponiveness with methacholine or histamine. Enhanced inflammatory activity has been proposed to play a role in the development of nocturnal asthma. Therefore, assessment of indirect hyperresponsiveness within one day might provide more insight into the pathogenetic mechanisms leading to increased nocturnal airways constriction. Little is known about the effects of repeated propranolol inhalation tests within the same day, either on baseline FEV_1 or on propranolol airways responsiveness. Measurement of propranolol airways responsiveness appears to be reproducible from day to day when these tests are repeated within a time interval of 1 week (2, 3). However, it has been found that the decrease in FEV_1 after propranolol challenge is long-lasting, not recovering to within 5 percent of baseline values after 90 minutes (4). Furthermore, upon oral treatment with propranolol, the inhaled

bronchodilator response with isoprenaline was significantly limited (5). As a circadian variation in serum adrenaline levels has been proposed to underlie variations in airway diameter (6, 7), propranolol challenge tests may interfere with normal diurnal changes in airway diameter.

Previous challenge with a non-specific stimulus may either enhance or reduce the sensitivity to the same or another stimulus. Upon oral treatment with propranolol, an enhanced responsiveness to histamine has been observed in asthmatic subjects (8). On the other hand, a decreased airways constrictive response or tachyphylaxis has been reported after repeated inhalation with a variety of airway constrictive stimuli (9-12). It has been demonstrated that tachyphylaxis upon airway challenge can even last up to 6 hours (9,10). Up to now, tachyphylaxis upon propranolol inhalation has not been investigated.

The aim of this study was to investigate the effects of repeated standardized propranolol inhalation provocation tests within one day on normal diurnal variation in FEV_1 and on subsequent propranolol airways responsiveness in atopic asthmatic subjects. In diurnally active subjects, the lowest FEV_1 is reached in the early morning, whereas the highest value is found in the afternoon. Therefore, propranolol challenges were performed with an interval of at least eight hours at 8 AM and 4 PM on the same study day and on a control day at 4 PM.

Methods

Subjects

Fifteen atopic asthmatic subjects were recruited from the outpatient clinic of our department of pulmonary diseases and through advertisements in local newspapers. The study had been approved by the Ethics Committee of our hospital. All subjects signed an informed consent agreement. They had a history of episodic dyspnea or wheezing consistent with the clinical diagnosis of asthma (13) and had no concomitant diseases. Further selection criteria were: 1. age between 18 and 45 yrs; 2. atopy for housedust mite (HDM): positive intracutaneous test, expressed as histamine equivalent wheal size (HEWS) $\geq$ 0.9 (Diephuis Laboratories, Groningen, the Netherlands) or elevated specific IgE (RAST score $\geq$ 2 = 0.7 U/ml; Phadezym^R RAST Pharmacia, Uppsala, Sweden); 3. FEV_1 > 1.25 l and > 60 % predicted; post-bronchodilator FEV_1 (after salbutamol 200 mcg + spacehaler) $\geq$ 80 % predicted; 4. airways hyperresponsiveness to histamine, defined as the provocative concentration of

histamine that caused a 20% fall in FEV_1 (PC_{20}) of $\leq$ 32 mg/ml (30'' inhalation, 2' interval); 5. No use of oral corticosteroids or inhaled corticosteroids in a dose above 800 mcg daily within 2 months prior to the study.

Study design

The subjects attended the laboratory on two days. On control day A, spirometry and propranolol inhalation provocation tests were performed at 16.00 hours, on study day B at 08.00 and 16.00 hours. These days were planned in a randomized order and separated by 1 to 5 days. Inhaled bronchodilators were withheld from 12 PM the days before testing.

Propranolol inhalation - provocation tests

Initial spirometry was performed using a calibrated water-sealed spirometer (Lode B.V., Groningen, the Netherlands), according to standardized guidelines (14). Prechallenge FEV_1 was measured until three reproducible recordings were obtained. The best of three was used for analysis. Highest values were used for baseline prechallenge values. Inhalation provocation tests were performed according to a 2-minute tidal breathing method adapted from Cockcroft and coworkers (15). Solutions of propranolol HCl were freshly made every week from powder preparations (Bufa Chemie, Uitgeest, The Netherlands). After inhalation of 0.9% sodium chloride solution, doubling concentrations of propranolol (0.5 to 32 mg/ml in normal saline) were administered at room temperature as aerosols delivered by a DeVilbiss 646 nebulizer (DeVilbiss Health Care Inc., Somerset, PA), connected to the central chamber of an inspiratory and expiratory valve box with an expiratory aerosol filter. Solution output was 0.13 ml/min, while the air pressure control was adjusted to 1 atmosphere. The challenge was discontinued when FEV_1 had fallen $\geq$ 20 % from the prechallenge level. PC_{20} was calculated by linear interpolation of the last two data points of the log concentration - response curve.

Data analysis

Values are presented as mean (SD). PC_{20} values were analysed after base 2 logarithmic transformation. After verifying the normal distribution of values, differences in prechallenge FEV_1 and PC_{20} values at the different days and time points were analysed using Student's paired, two-tailed t-test. Correlations between variables were performed with

the Pearson correlation test. Values of $p \leq 0.05$ were considered statistically significant. All analyses were performed with the SPSS/PC+ V4.0 software package (SPSS Inc., Chicago, IL).

Results

Subjects

Subject characteristics and medication use are listed in table 1. Thirteen of the 15 subjects were nonsmokers. Mean FEV_1 was 90.3 $\pm$ 3.2 %. Airways hyperresponsiveness to histamine ranged from mild to severe (0.11 - 32.00 mg/ml), with a geometric mean value of 2.2 mg/ml.

Table 1. Details of participating subjects

Subject No.	Gender	Age (yr)	Medication use*	HEWS HDM	RAST HDM score	FEV_1 % Pred	PC_{20} histamine (mg/ml)
1	M	26	CS	nd	2	112	32.00
2	M	28	B, A, CS	nd	4	97	8.00
3	M	29	CS	1.0	4	107	0.36
4	M	32	-	1.2	2	105	16.00
5	M	18	B, Cr	1.2	3	86	2.88
6	F	21	B	nd	4	96	3.73
7	M	20	A	1.2	2	89	4.00
8	M	26	B, CS	1.7	4	86	0.12
9	F	22	B, CS	1.1	3	91	0.55
10	M	20	-	nd	3	76	1.24
11	M	34	-	1.2	3	75	2.54
12	F	40	B	0.9	0	94	12.70
13	F	38	A, Cr, CS	1.5	4	87	0.11
14	M	36	B, Cr, CS	1.6	2	65	2.00
15	F	43	-	1.2	1	89	2.73

* A = anticholinergic, B = β_2-adrenergic, Cr = cromoglycate and CS = corticosteroid inhalations.

Prechallenge FEV_1

In 13 individuals the FEV_1 values at 16.00 hrs on study day B were lower as compared with the values at 16.00 hrs on control day A. In subjects no. '5' and '12' the FEV_1 values were unaltered. No clear difference in subject characteristics or treatment could be detected distinguishing these subjects from the others. Mean FEV_1 at 16.00 hrs on day B, eight hours after the previous propranolol challenge, was significantly lower as compared to the mean value at 16.00 hrs on control day A (3.38 $\pm$ 0.23 vs. 3.70 $\pm$ 0.24 l, respectively; $p = 0.001$) (figure 1). Mean FEV_1 at 08.00 hrs on day B was also significantly lower than the value at 16.00 hrs on day A (3.42 $\pm$ 0.42 vs. 3.70 $\pm$ 0.24 l;$p = 0.001$), which can be ascribed to the circadian fluctuation in FEV_1. Mean FEV_1 values at 08.00 and 16.00 hrs on day B were not significantly different.

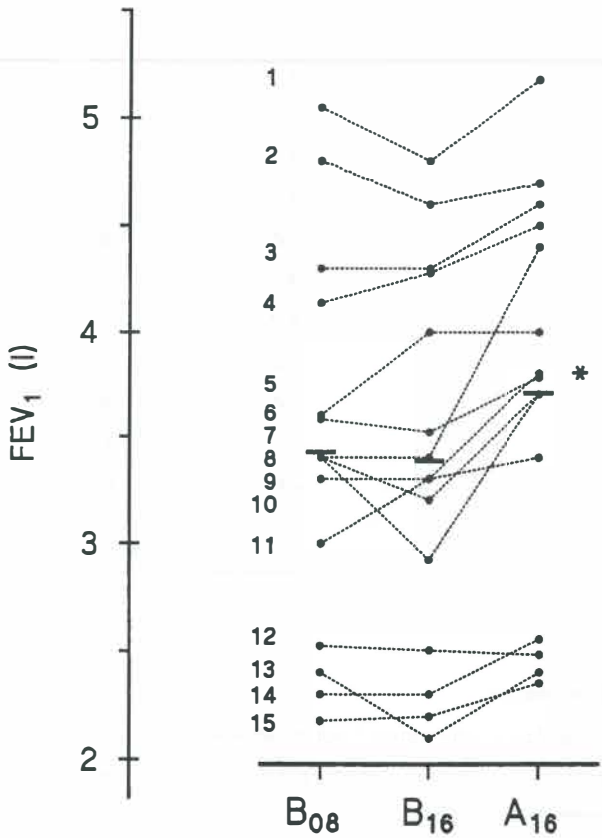

Figure 1. Prechallenge FEV_1 at the study day at 08.00 hrs (B_{08}) and at 16.00 hrs (B_{16}: eight hours after propranolol challenge) and at the control day at 16.00 hrs (A_{16}). Each set of dots represents one individual. The numbers on the left are the subject numbers. Mean values are shown by horizontal bars. * $p = 0.001$ compared with the other time points.

PC_{20} propranolol

All subjects demonstrated airways hyperresponsiveness to propranolol (table 2). Twelve subjects responded to the higher provocation concentrations, in the range between 8 and 32 mg/ml. No significant differences were found between geometric mean PC_{20} values measured at the three time points. No relationship was found between $\log_2 PC_{20}$ propranolol values at the control day and $\log_2 PC_{20}$ histamine values ($r = -0.03$, NS).

Table 2. Airways responsiveness to propranolol (mg/ml)

Subject	Day B		Day A
no.	08.00	16.00	16.00 hrs
1	11.3	24.8	16.8
2	9.0	9.0	18.0
3	17.0	12.0	11.0
4	20.8	20.8	11.7
5	14.0	16.0	13.0
6	9.2	22.2	25.6
7	31.0	19.0	20.0
8	21.1	21.2	20.0
9	1.4	0.8	4.1
10	16.0	13.0	11.0
11	16.0	19.0	9.0
12	6.0	7.7	1.2
13	9.0	9.0	9.0
14	1.0	1.0	5.0
15	6.6	12.4	16.0
geometric mean	9.3	11.3	9.3

Discussion

This study was carried out to investigate the effects of a propranolol inhalation provocation test on baseline FEV_1 and propranolol airways responsiveness in atopic asthmatic subjects within the same day. It demonstrates that in 13 out of 15

asthmatics, eight hours after propranolol provocation, the baseline FEV_1 had still not recovered to values comparable with those measured at the control day. The mean difference was 9%, which is more than the day to day variation of FEV_1, known to be within 4% (16). This indicates that propranolol provocation had a long-lasting effect on FEV_1, thereby opposing the normal diurnal increase in airway diameter. Furthermore, no effects on propranolol airways responsiveness were found.

Propranolol is regarded as a stimulus indirectly inducing airways constriction (1). It acts by blockade of ß-adrenergic receptors, which are present on many different cell types located in the airway walls, including bronchial smooth muscle cells, cholinergic and non-cholinergic non-adrenergic (NANC) nerves and mast cells (17). Beta-adrenergic stimulation induces relaxation of airways smooth muscle cells, along with inhibition of mast cell mediator release and inhibition of the cholinergic and NANC neurotransmission. Since propranolol induces airways constriction in asthmatic but not in non-asthmatic subjects, it has been proposed that a dysfunction of inhibiting M_2 - autoreceptors on cholinergic neurotransmission exists in asthma (18). As a result of the counteraction of propranolol on the inhibiting ß-adrenergic stimulation on cholinergic neurotransmission, acetylcholine release increases. In non-asthmatic subjects this leads to an increased negative feedback on cholinergic neurotransmission via M_2 - autoreceptors, but in asthmatics the inhibition by M_2 -receptors can be impaired as a consequence of interference by inflammatory mediators. Evidence for an indirect action on the cholinergic neurotransmission is supported by the protective effect of oxitropium bromide and atropine on propranolol induced airways obstruction (19, 3). Besides, involvement of the NANC neurotransmission is supported by the finding that inhalation of vasointestinal peptide decreased propranolol airways responsiveness (20). Finally, pretreatment with cromoglycate protected against propranolol induced airways obstruction, suggesting involvement of mast cell release or axon reflex mechanisms (21).

The contribution of the ß-adrenergic system to the diurnal rhythm in airway tone has been suggested before, however contradicting results have been found (6, 7, 22). Circulating adrenaline, produced by the adrenal medulla, is the major stimulus of ß-receptors in the airways. A circadian variation in adrenaline serum concentration exists along with the decrease in early morning airway diameter, being lowest at 4 AM and increasing in the daytime (6, 7). Momentaneous correction of the nocturnal fall in plasma adrenaline at 4 AM to 4 PM levels in patients with nocturnal asthma did,

however, not improve nocturnal lung function (22). The authors therefore stated that the nighttime fall in plasma adrenaline is not a sole cause of nocturnal asthma. Results of our study, showing that ß-receptor blockade by inhaled propranolol opposed the improvement of the daytime FEV_1, nevertheless favours a role for circulating adrenaline in circadian variation of airway diameter. A more sustained ß-adrenergic stimulation in the in vivo situation by increasing serum adrenaline concentrations during daytime may modulate the bronchomotor tone, e.g. by inhibiting the inflammatory triggering of vagal or NANC reflex mechanisms. As cromoglycate treatment has been shown ineffective in preventing early morning dipping of PEFR in patients with nocturnal asthma (23), ß-adrenergic inhibition of mast cell release may be of minor importance.

Results of this study also show that propranolol inhalation can be repeated within the same day with an 8 hours interval without affecting propranolol airways responsiveness. Investigations on occurence of altered sensitivity upon propranolol inhalation have so far not been described, but have been reported for many other stimuli when repeated tests were performed. An increased sensitivity to histamine inhalation was found 2 hours after oral intake of 40 mg propranolol by asthmatic subjects (8). Tachyphylaxis has been demonstrated after repeated inhalation provocation tests with various direct and indirect acting stimuli, such as histamine, methacholine, adenosine 5'- monophosphate and bradykinin (9- 12). Tachyphylaxis to histamine and adenosine 5'- monophosphate was found to last up to 6 hours (9, 10). Generally, tachyphylaxis is more prone to develop when the concentration of the inhaled stimulus is higher and time intervals between the challenge tests are shorter. Although in our study most subjects were challenged with higher propranolol concentrations, no tachyphylaxis occurred.

In conclusion, this study demonstrates that propranolol inhalation opposes the normal diurnal increase in FEV_1 in most asthmatic subjects. This favours a role for serum adrenaline in daytime modulation of the bronchomotor tone, e.g. by inhibiting the inflammatory triggering of vagal or NANC reflex mechanisms. In assessing indirect airways responsiveness within the same day it must be taken into account that the effect of propranolol inhalation on FEV_1 lasts up to eight hours and makes propranolol challenge tests less suitable for studying indirect airways responsiveness within one day. Other indirectly acting stimuli are probably more convenient. Furthermore, repeated inhalation provocation tests with propranolol within a time interval of eight hours have no effects on airways responsiveness to propranolol.

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Chapter 5

INFLAMMATORY CELL NUMBER AND MEDIATORS IN BRONCHOALVEOLAR LAVAGE FLUID AND PERIPHERAL BLOOD IN ASTHMATIC SUBJECTS WITH INCREASED NOCTURNAL AIRWAYS NARROWING

Ytske Oosterhoff, Henk F. Kauffman, Bea Rutgers, Freek J. Zijlstra,
Gerard H. Koëter, Dirkje S. Postma

Submitted

Abstract

Background: Increased nocturnal airways narrowing (NA) in asthma is thought to occur as the result of intensification of inflammatory processes in the airways. In this study we investigated the presence of inflammatory cells and mediators in bronchoalveolar lavage (BAL) fluid and peripheral blood (PB) and assessed their relationship with the occurrence of increased NA.

Methods: BAL and PB sampling were assessed at 16.00 and 04.00 h, seperated by $\geq$ 7 days, in 8 nonatopic healthy subjects (Group 1) and 17 atopic asthmatic subjects, using inhaled bronchodilators only. They were prospectively assigned to groups with and without NA, as defined by a mean circadian PEF-variation $<$ 15% (Group 2) and $\geq$ 15% (Group 3).

Results: Significantly higher eosinophil numbers and inflammatory activation products (ECP, EDN, histamine) were found in BAL fluid and PB from asthmatic subjects in comparison with controls. However, increased NA was not generally associated with a circadian fluctuation in cell number and inflammatory mediators in BAL fluid and PB. No differences in inflammatory cell numbers were found that distinguished between Groups 2 and 3. However, in Group 3, significantly higher BAL prostaglandin D_2 (PGD_2) levels (70 [28-102] vs. 24 [11-90] pg/ml; $p = 0.04$) and serum ECP levels (17.6 [6.3-17.5] vs. 16.1 [6.3-60.3] ng/ml; $p = 0.03$) at 16.00 h were detected as compared with Group 2.

Conclusions: Our findings suggest that increased NA is more likely to occur in asthmatic subjects with ongoing increased cellular activation at daytime.

Introduction

Increased nocturnal airways narrowing in asthma is thought to occur as the result of intensification of inflammatory processes in the airways of subjects with nocturnal asthma, as has been suggested by an increase in (indirect) airways hyper-responsiveness at night (1) and findings that adequate anti-inflammatory treatment with inhaled corticosteroids reduced the circadian variation in airways obstruction and responsiveness (2).

Inflammatory conditions may be associated with an increased influx of cells into the airway wall, along with marked shifts in cell populations in the bronchoalveolar lavage (BAL) fluid. So far, however, conflicting results have been reported on nocturnal increases in cell numbers and morphology, as analysed in BAL fluid from subjects with

nocturnal asthma (3, 4). In addition, peripheral blood (PB) eosinophil numbers and their activation parameters during the night in nocturnal asthmatic subjects have been found either increased (5, 6) or decreased (7) and conflicting results have been reported on PB histamine levels as well (7, 8). From the various studies it can often not be deduced whether the circadian variations are due to pathologic processes underlying nocturnal asthma or whether they belong to normal physiological circadian rhythms. For instance, both in normal and asthmatic subjects circadian rhythms exist in PB leukocyte number and their functional activity (9). Similar chronobiological rhythms in cell number and activity in the airways can be postulated. So far, however, circadian variations in inflammatory cells in BAL fluid of healthy individuals have scarcely been investigated.

The aim of this study was to investigate the presence of inflammatory cells and mediators in BAL fluid and PB at 16.00 and 04.00 h from healthy and atopic asthmatic subjects and to determine their relationship with the occurrence of increased nocturnal airways narrowing in asthma, as assessed by measurement of the circadian PEF-rhythm. Eosinophils and mast cells are considered as principal effector cells (10, 11). Their activation parameters, as assessed by EG_2-positive staining cells, eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), histamine and prostanoids, have been found increased in the BAL fluid of stable and symptomatic asthmatic subjects (12-16). These cell types and activation parameters were assessed in BAL fluid, whereas in PB eosinophil numbers and ECP levels were determined.

Materials and methods

Subjects

Eight healthy and 17 asthmatic subjects (18-45 yrs) participated in the study (table 1). The subjects were recruited through advertisements in local newspapers and from the outpatient clinic of our hospital. The study was approved by the Ethical Committee of the hospital, and written informed consent was given by each subject.

At entry to the study the selected *healthy volunteers* had: 1. no history of lung disease or allergy; 2. no atopy: negative intracutaneous tests against 18 common aeroallergens, expressed as histamine equivalent wheal size (HEWS) < 0.7 (Diephuis Laboratories, Groningen, the Netherlands); 3. no airways response upon provocation with methacholine: < 10% fall in forced expiratory volume in one second (FEV_1) after inhalation of methacholinebromide in a concentration of 9.8 mg/ml (2' inhalation, 5' interval); 4. no upper respiratory infections within 1 month before the study and no

concomitant diseases.

Asthmatic subjects were prospectively selected on the basis of: 1. a history of episodic dyspnea or wheezing consistent with the clinical diagnosis of asthma (17); 2. atopy: positive intracutaneous tests against housedust mite or two other of 18 tested common aeroallergens (HEWS $\geq$ 0.7); 3. FEV_1 > 1.50 l and $\geq$ 70% predicted; post-bronchodilator FEV_1 (salbutamol 400 μg using a spacehaler) $\geq$ 80 % predicted; 4. airways hyperresponsiveness to methacholine, defined as the provocative concentration of methacholinebromide that caused a 20% fall in FEV_1 (PC_{20}) of $\leq$ 9.8 mg/ml (2' inhalation, 5' interval); 5. no use of oral corticosteroids within 2 months before the study; 6. no upper respiratory infections within 1 month before the study and no other concomitant diseases.

Study design

In the asthmatic subjects, inhaled corticosteroids and cromolyns were stopped four weeks before the start of the study. Until the last bronchoscopy they were allowed to use inhaled bronchodilators only (β_2-agonists or ipratropiumbromide).

The week before the study, PEF values were recorded during three days at 08.00, 12.00, 16.00, 20.00, 24.00 and 04.00 h. Bronchodilators were withheld. The best of three PEF maneuvers was recorded. The mean circadian PEF-variation of the three days was defined as: (highest - lowest 24 h value)/mean 24 h value. Subjects were prospectively selected in three groups: *Group 1* consisted of the healthy volunteers, whereas the asthmatic subjects were assigned to *Group 2* if the mean circadian PEF-variation was < 15% and to *Group 3* if the PEF-variation was $\geq$ 15%. Nocturnal asthma symptoms were scored on the basis of nocturnal awakening due to nocturnal dyspnea experienced in the last week: 0 = never, 1 = sometimes, 2 = every night. FEV_1 and airways hyperresponsiveness to methacholine were assessed within 5 days before the first bronchoscopy.

Thirty minutes before each bronchoscopy, FEV_1 measurements were performed and PB samples were taken. Subjects rested during 15 minutes before the lung function measurements in daytime. At night, subjects were admitted to the hospital at 22.00 h and were awakened 15 minutes before the measurements. Bronchodilator therapy was withheld 8 h before the pulmonary function tests. Bronchoscopy was performed at 16.00 h (4:00 P.M.) and 04.00 h (4:00 A.M.) on two different days, separated by at least seven days. Nineteen subjects underwent their first bronchoscopy at 16.00 h, five

at 04.00 h.

Pulmonary function and inhalation provocation tests

PEF values were recorded with a Wright mini peak flow meter. FEV_1 was measured with a calibrated water-sealed spirometer according to standardized guidelines (18), the best of three measurements being used for analysis. Airways responsiveness to methacholine was measured using a 2-minute tidal breathing method (1). PC_{20} values were calculated by linear interpolation of the last 2 points of the log concentration response curve.

Bronchoscopy procedure

Fiberoptic bronchoscopy was undertaken according to guidelines described by the American Thoracic Society (20). Premedication consisted of intramuscular injection of 0.5 mg atropine and inhalation of 200 μg salbutamol via a spacehaler. Lidocaine 4% was applied as local anaesthesia into upper airways and bronchial tree. Supplementary oxygen was available. Bronchoscopy was performed using an Olympus B1 IT10 flexible fiberoptic bronchoscope (Olympus Optical Co., Tokyo, Japan). The instrument was inserted through the mouth and wedged in the lateral segment of the right middle lobe. Subsequently, 10 aliquots of 20 ml sterile phosphate - buffered saline (PBS) of 37 °C were instilled and recovered by gentle suction (-40 cm H_2O) after each aliquot. The BAL fluid was collected in polypropylene tubes, which were immediately placed in ice.

Laboratory techniques

The BAL fluid was pooled in two fractions: pool 1 consisted of the fluid recovered from the first 2 x 20 ml PBS instilled, containing constituents from the proximal airways, pool 2 the fluid from the remaining 8 x 20 ml. Cell numbers and activation parameters were assessed in the first fraction. Due to a limited amount of BAL fluid in this fraction, the eicosanoids had to be determined in pool 2. The lavage fluid was filtered through a 100 μm pore filter of a venous infusion system (Curapharm, Medica B.V., Hospital Supplies, The Netherlands) to remove mucus, and centrifuged at 400 g at 4 °C for 5 minutes. The BAL supernatant was stored at -80 °C until further determination. The cell pellets were resuspended in 1 ml PBS with 0.5% heat - inactivated bovine serum albumin (PBS/BSA 0.5%).

Total leukocyte numbers in PB and BAL cell suspensions were counted in a

Coulter Counter (Model S-plus VI; Coulter Electronics, Hialeah, USA), and viability was assessed by cellular exclusion of trypan blue. BAL cytospin preparations of both pools were made from 100 μl fractions of the BAL cell suspension, diluted to a volume of 0.25 x 10^6 cells/ml in PBS/BSA 0.5%, using a Shandon cytospin-2 centrifuge (Shandon Inc, Pittsburgh, USA). After staining with May-Grünwald-Giemsa, the average of 300 cell counts on two slides was taken as the differential cell count. Additional cytospins were sealed in plastic and stored at - 80 °C for future staining and analysis. PB eosinophil numbers were counted in a Bürker chamber after staining with eosine.

Activated eosinophils in the BAL fluid were identified by immunostaining of acetone - fixed cytocentrifuge preparations with the monoclonal antibody EG_2 (Sanbio, Uden, The Netherlands), which detects a cleaved part of the eosinophilic cationic protein. A two - step alkaline phosphatase procedure was performed, according to the method described by Tai and coworkers, with minor modifications (21). The slides were incubated with 50 μl EG_2 solution (1:50) for 60 minutes. As a control for false-positive counting the primary antibody was omitted or an irrelevant antibody was used as a first step. The slides were then incubated for 30 minutes with an alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody (1:40) (Sigma, St Louis, USA) supplemented with 5% human AB serum, followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:40) supplemented with 5% human AB serum. The phosphatase reactivity was demonstrated by Fast Red (Sigma) (1 mg/ml) in Tris-HCl buffer 0.1M pH 8.2, containing 0.2 mg/ml naphtol AS-MX (Sigma) with 2mM Levamisole (Sigma) to inhibit endogenous alkaline phosphatase. The slides were counterstained with fresh Mayers' Haematoxylin. EG_2^+ cell counts were performed on two slides and the average counts of 500 cells on each slide was taken as the definitive number of positive cells. Only intact and unclustered cells were counted. Total EG_2^+ cell counts were calculated from the total leukocyte number/ml BAL fluid.

ECP and EDN determination in serum and BAL fluid. Serum was obtained after coagulation of the blood for one hour at room temperature. The supernatant was centrifuged twice for 10 minutes at 2000 g and stored at -80° C until analysis. ECP was measured in serum, whereas ECP and EDN were determined in unprocessed BAL supernatants from pool 1. This was performed by double antibody radioimmunoassays (RIA) (Pharmacia Diagnostics AB, Uppsala, Sweden), as described previously (22, 23). The detection level in both assays was 0.1 ng/ml.

Histamine determination in BAL fluid. Histamine was determined in unprocessed lavage supernatants from pool 1, using the RIA procedure from Immunotech (Maine, USA), in accordance with the manufacturer's protocol.

Eicosanoid determination. Immediately after the BAL procedure, 20 ml of BAL supernatant from pool 2 was processed on C_{18} SepPak cartridges (Millipore, Milford, USA) as described previously (24), eluted with 2.5 ml methanol and stored at - 80 °C until analysis. Samples of 200 μl eluted fluid were pipetted into polypropylene tubes and dried with a Savant sample concentrator. After dissolving in 300 μl assay buffer, thromboxane B_2 (TxB_2) was determined by means of a [^{3}H] RIA using antisera from Advanced Magnetics Inc. (Ma, USA) and [^{3}H] labelled compounds from Amersham UK; prostaglandins PGD_2 and $PGF_{2\alpha}$ were [^{3}H] kits obtained from Amersham, UK (24). 6k$PGF_{1\alpha}$ was determined by means of a [^{125}I] RIA kit (Du Pont, Germany). Leukotriene $C_4/D_4/E_4$ (LTC_4) was measured at room temperature in a microtiter enzyme immunoassay according to protocol (Biotrak, Amersham, UK)(24). Cross - reactivities for each assay to related compounds were negligible or less than 2% at B/Bo 50%.

Data analysis

PC_{20} values were analysed after base 2 logarithmic transformation and expressed as the geometric mean PC_{20} value. After verifying the normal distribution, changes in values of lung function parameters within subjects were analysed using Student's paired two-tailed t-test and between groups using Student t-test for unpaired observation. Non-parametric tests were used to analyse the cell number and activation parameters, the number of positive skin tests and nocturnal asthma score. The Mann-Whitney U test was used to compare data between groups, and Wilcoxon's matched signed rank test was applied for within-group analyses. Correlations between the circadian PEF-variations and lung function or cell parameters were made using Spearman's rank correlation tests. All analyses were performed with the SPSS/PC$^+$ V 4.01 software package (SPSS Inc., Chicago, IL). Values of $p \leq 0.05$ were considered statistically significant.

Results

Subjects

Subject characteristics are listed in table 1. Group 1 consisted of the healthy

Table 1. Subject characteristics.

No.	Age (yr)	Gender (M/F)	Smoking	Med[1]	Skin tests[2]	FEV_1% pred.	PC_{20} Meth. mg/ml	NA score[3]	PEF-var%[4]
Group 1 Healthy controls									
1	44	M	-	-	0	121	>9.8	-	4
2	21	F	-	-	0	107	>9.8	-	6
3	20	M	-	-	0	88	>9.8	-	8
4	32	M	-	-	0	92	>9.8	-	8
5	27	F	-	-	0	99	>9.8	-	9
6	21	M	-	-	0	116	>9.8	-	9
7	34	F	-	-	0	125	>9.8	-	11
8	22	F	-	-	0	93	>9.8	-	11
Group 2 ASTHMA circadian PEF rhythm < 15%									
1	30	M	-	B	7	100	1.33	0	6
2	20	M	-	B	6	105	1.88	0	8
3	19	M	-	CR	6	80	1.64	0	10
4	22	F	-	B	7	90	0.34	1	11
5	18	M	-	B + CS	6	82	0.63	0	12
6	29	F	-	B + CS	6	97	1.68	0	13
7	40	M	-	B	7	94	0.36	1	14
Group 3 ASTHMA circadian PEF- rhythm ≥ 15%									
1	41	M	-	A	2	114	0.73	0	19
2	22	M	-	B + CS	8	90	0.66	1	20
3	22	F	-	B + CS	7	69	0.04	1	23
4	32	F	-	A + CS	3	94	0.60	1	24
5	29	M	-	B	4	80	0.64	0	25
6	21	F	-	B	2	98	0.96	1	28
7	21	F	+	B	8	68	0.08	1	29
8	26	F	+	B + CS	8	82	0.17	1	31
9	41	F	-	B + CS	7	92	0.37	2	44
10	20	F	-	B + CR	5	88	0.04	2	46

1. Regular inhaled therapy until 4 weeks before participation in the study: A = anticholinergic, B = $ß_2$ adrenergic, CR = cromoglycate and CS = corticosteroid;
2. Number of positive skin tests;
3. Awakening because of nocturnal dyspnea : 0 = never, 1 = sometimes, 2 = every night in previous two weeks;
4. Expressed as: (highest - lowest 24 h value)/mean 24 h value.

volunteers. Seven asthmatic subjects were assigned to Group 2 (mean circadian PEF-variation < 15%) and 10 to Group 3 (mean circadian PEF-variation $\geq$ 15%). Mean circadian PEF-rhythms in the control group and Group 2 were not significantly different from each other. Age and gender were not significantly different between the three groups, nor were the number of positive skin tests and mean FEV_1% predicted between the two asthma groups. Mean FEV_1% predicted was significantly lower in both asthma groups as compared with the healthy control group ($p < 0.05$).

Asthmatic subjects in Group 3 scored significantly higher on awakening due to nocturnal dyspnea ($p = 0.03$), and their geometric mean PC_{20} methacholine was significantly lower as compared to the asthmatic subjects in Group 2 (0.26 and 0.91 mg/ml respectively; $p = 0.02$). The circadian PEF-variation of all asthmatics was significantly correlated with the PC_{20} methacholine values ($r = -0.65$, $p = 0.007$) and with the nocturnal asthma symptom score ($r = 0.73$, $p = 0.009$).

FEV_1 measurements

Mean FEV_1 in Group 3 fell significantly during night as compared with the 16.00 h value (from 87.5 $\pm$ 13.7 to 75.7 $\pm$ 19.2 %; $p = 0.005$), whereas this was less pronounced in Group 2 (from 92.6 $\pm$ 9.2 to 88.1 $\pm$ 10.1%; $p = 0.09$). In the healthy control group no differences were found between the 16.00 and 04.00 h values (105 $\pm$ 14.2 and 105 $\pm$ 13.2%, respectively).

Bronchoscopy procedure

Results of BAL parameters in Group 3 are based on data from 9 subjects, because subject number 4 in this group refused a second bronchoscopy.

BAL cell analysis.

The volume of fluid recovered, total and differential cell numbers in pool 1 are shown in table 2. The volume of fluid recovered was lower in the asthmatic groups than in the normal volunteers, but this only reached significance at 04.00 h ($p = 0.04$). Mean total and differential cell numbers/ ml BAL fluid were not significantly different among the various groups at the different time points.

Eosinophil numbers were higher in the asthmatic subjects as compared to the control group (16.00 h: $p \leq 0.004$; 04.00 h: $p = 0.05$), but were not significantly different between the two asthmatic groups. In Groups 1 and 2, they tended to

Table 2. Cellular aspects of BAL fluid (pool 1). Medians with range.

	Group 1 Controls		Group 2 Asthma PEF-rhythm < 15%		Group 3 Asthma PEF-rhythm ≥ 15%	
	16.00	04.00	16.00	04.00	16.00	04.00 h
Recovery %	48.8 (37.5-57.5)	47.5 (40.0-57.5)	45.0 (25.0-57.5)	36.3 (22.5- 50)[1]	40.0 (25.0- 55)	32.5 (17.5-50)[1]
Total leukocytes x 10^3/ml	50.4 (8.6-199)	64.3 (28.0-87.9)	48.9 (26.5-86.7)	68.5 (30.7-164)	51.8 (25.5-155)	71.0 (17.8-249)
Alveolar macrophages	48.8 (7.7-193)	56.7 (24.6-72.8)	46.1 (24.6-79.5)	64.4 (18.4-146)	50.4 (19.5-151)	68.0 (15.2-171)
Mast cells	0.0 (0.0-0.04)	0.0 (0.0-0.1)	0.0 (0.0-0.05)	0.0 (0.0-0.0)	0.0 (0.0-0.2)	0.0 (0.0-0.0)
Lymphocytes	1.5 (0.1-2.6)	1.5 (0.0-3.5)	1.4 (0.6-2.0)	2.2 (0.7-6.0)	0.8 (0.0-2.7)	1.0 (0.1-2.9)
Neutrophils	0.7 (0.3-3.6)	2.9 (1.4-14.9)[2]	0.1 (0.0-1.2)	2.5 (0.0-6.5)[2]	0.5 (0.0-19.5)	0.2 (0.0-7.1)
Eosinophils	0.2 (0.0-0.8)	0.6 (0.0-5.0)	1.1 (0.5-4.6)[3]	2.6 (1.2-6.7)[3]	0.7 (0.2-4.0)[3]	1.5 (0.1-75.1)
EG_2^+ cells	0.1 (0.0-0.2)	0.2 (0.0-2.6)	0.6 (0.1-6.7)[4]	2.3 (0.7-14.7)[4]	0.6 (0.1-2.0)[4]	1.0 (0.0-16.7)
Epithelial cells	0.1 (0.0-1.0)	0.1 (0.0-3.3)	0.0 (0.0-2.1)	0.1 (0.0-0.2)	0.1 (0.0-2.5)	0.2 (0.0-2.8)

1 all asthmatic subjects: $p = 0.04$ compared with 16.00 h value; $p = 0.01$ compared with 4.00 h value of Group 1;
2 $p < 0.05$ compared with 16.00 h value within the group;
3 all asthmatic subjects, 16.00 h: $p \leq 0.004$ and 04.00 h: $p = 0.05$ compared with value of control group;
4 all asthmatic subjects, 16.00 h: $p < 0.001$ and 04.00 h: $p = 0.001$ compared with value of control group.

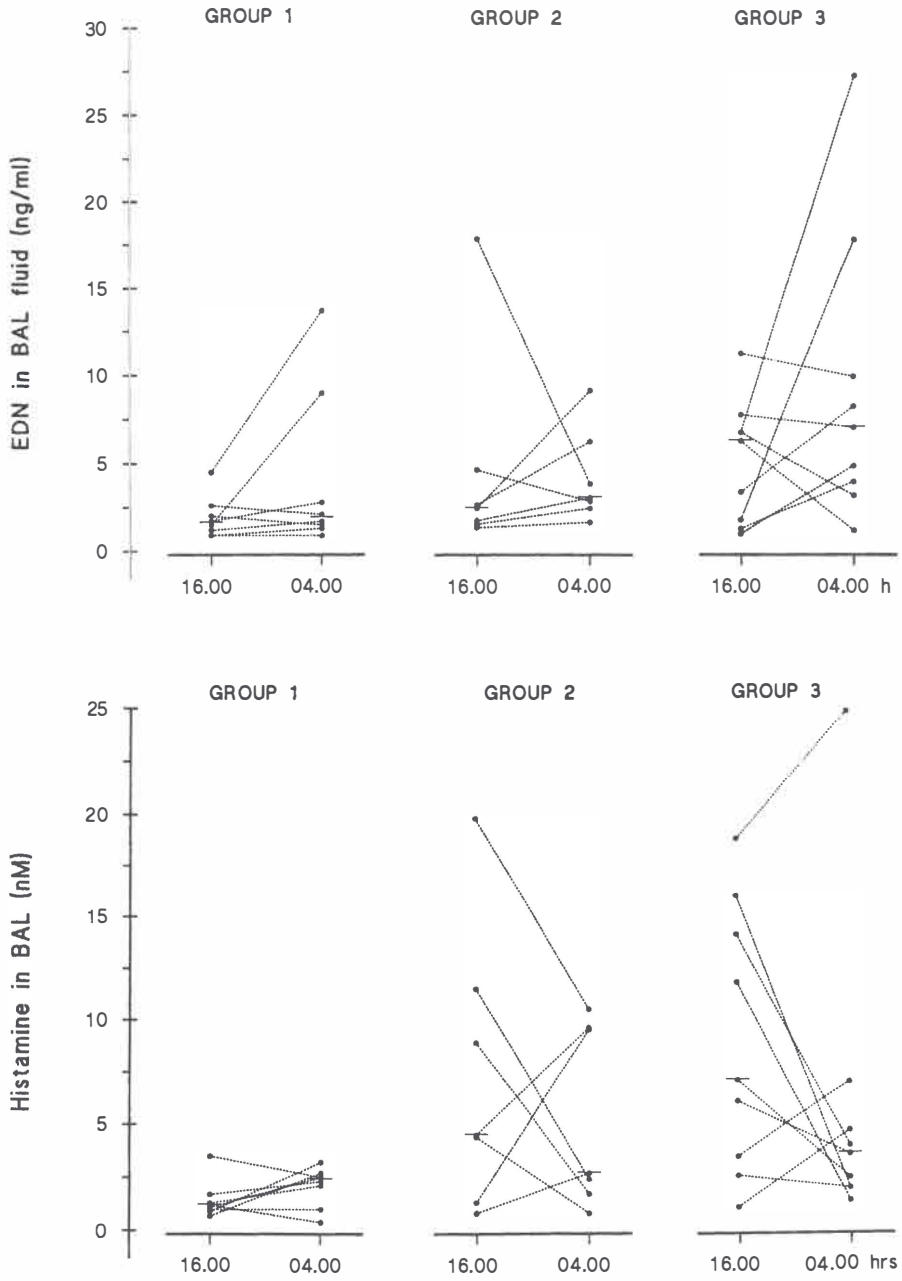


Figure 1. EDN (upper panel) and histamine (lower panel) levels in BAL fluid (pool 1) at 16.00 and 04.00 h in control subjects (Group 1), asthmatic subjects with circadian PEF-variation < 15% (Group 2) and ≥ 15% (Group 3). Individual values are represented by dots, median values by horizontals bars. For details, see *Results* section.

increase at night, running parallel with increased numbers of neutrophils at 04.00 h ($p = 0.02$ and $p = 0.03$, respectively).

EG_2^+ cell numbers showed the same pattern as the eosinophil numbers (table 2). The numbers were significantly higher in the asthmatic subjects than in the controls at 16.00 h ($p < 0.001$), with the same trend at 04.00 h. No statistical difference was found between both asthmatic groups. The numbers of EG_2^+ cells were always higher at 04.00 h than at 16.00 h, reaching significance in Group 2 ($p = 0.04$).

BAL inflammatory mediator levels.

In the asthmatic subjects *EDN and histamine* values were higher than in the control group, reaching significance at 16.00 h ($p = 0.04$ and $p = 0.003$, respectively) (figure 1). There was no statistically significant difference in EDN and histamine levels between the asthma groups at both time points. No significant variation in EDN and histamine levels between 04.00 and 16.00 h was found in the three groups. In contrast to EDN, most ECP levels were below the detection level of 0.1 ng/ml and were therefore not suitable to detect differences between the time points among the various groups.

Eicosanoid levels in BAL fluid are shown in figure 2. Due to the limited recovery of BAL fluid, eicosanoid levels could not be assessed in two asthmatic subjects, one from Group 2 and one from Group 3. Significantly higher levels of PGD_2 were found in Group 3 as compared to the other groups at both time points ($p \leq 0.04$). Furthermore, TxB_2 levels in Group 3 were significantly higher as compared to Group 2 at both time points ($p = 0.04$), but this did not reach significance as compared to the control group. Increased levels of LTC_4 were found in Group 3 at 04.00 h as compared to the other groups, reaching significance with the control group ($p = 0.03$). No significant differences in $PGF_{2\alpha}$ and $6kPGF_{1\alpha}$ levels were observed among the various groups. No significant variations in eicosanoid levels between 04.00 and 16.00 h within the three groups were found.

Peripheral blood analysis

Total leukocyte counts (table 3) did not show a statistically significant difference between normal subjects, all asthma subjects, or in the asthma subgroups at 16.00 and 04.00 h. Furthermore, there were no variations between 04.00 and 16.00 h in the three groups.

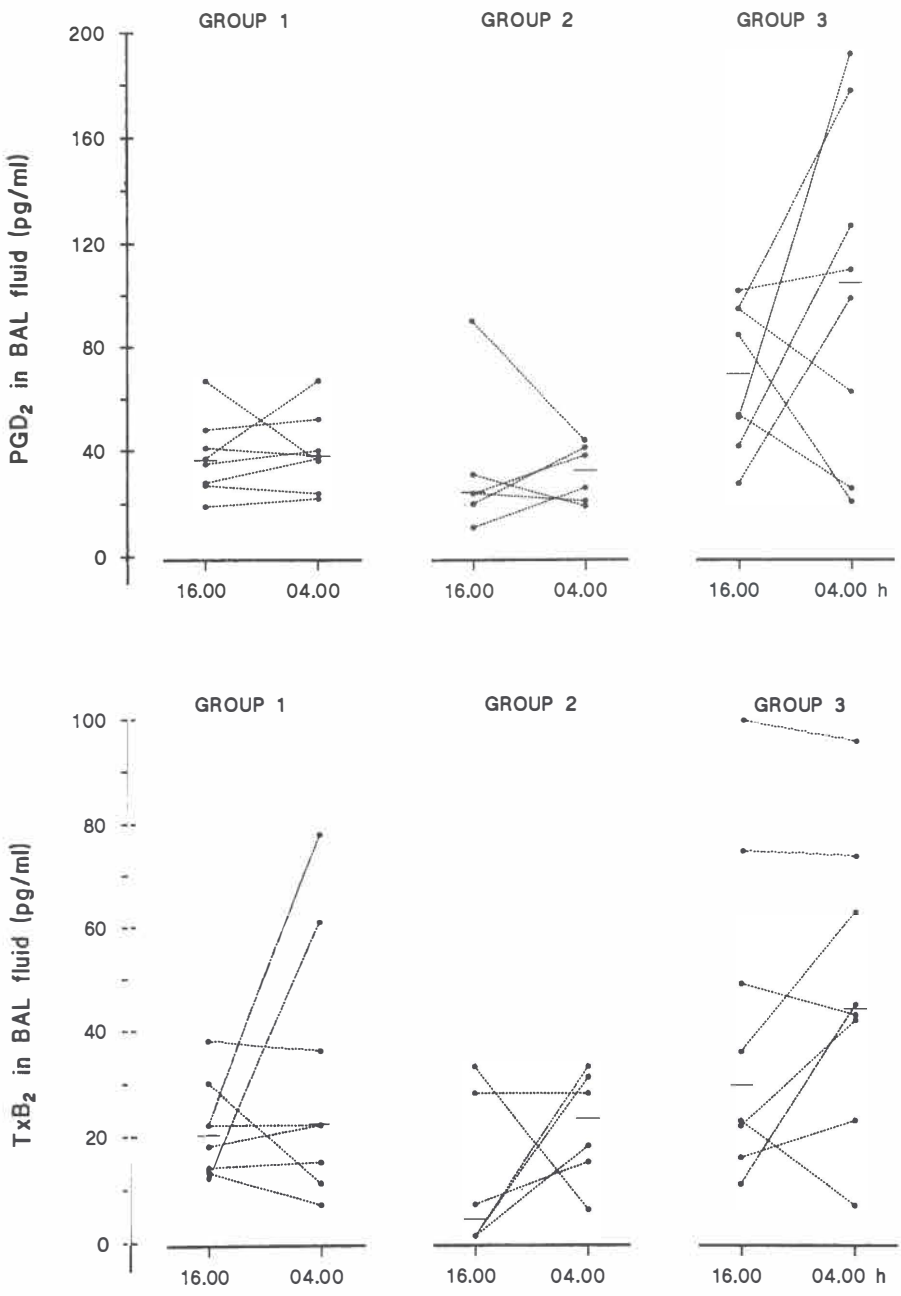
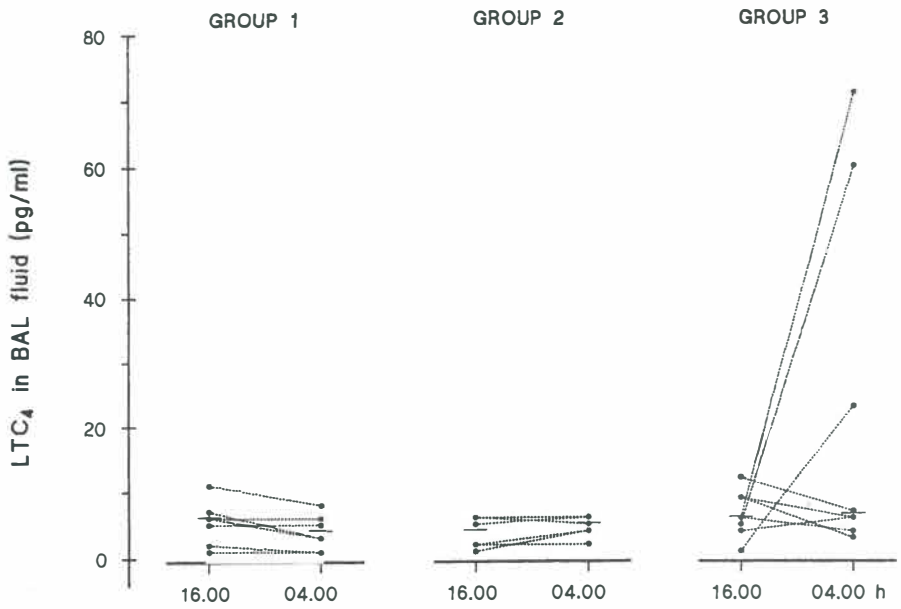


Figure 2. Eicosanoid levels in BAL fluid (pool 2) at 16.00 and 04.00 h in control subjects (Group 1), asthmatic subjects with circadian PEF-variation < 15% (Group 2) and ≥ 15% (Group 3). Individual values are represented by dots, median values by horizontals bars. For details, see *Results* section.



Eosinophil numbers in PB were significantly higher in all asthmatic subjects as compared to the control subjects at 16.00 h and 04.00 h ($p \leq 0.01$), but there was no significant difference between the two asthma groups. Mean total eosinophil numbers were increased in all groups at 04.00 h compared with the 16.00 h values. This reached significance in Group 2 ($p = 0.05$).

Mean *serum ECP* level at 16.00 h were not significantly different between Groups 1 and 2, while this level was significantly higher in Group 3 ($p \leq 0.03$). At 04.00 h, levels in both asthma groups were significantly higher than in the control group ($p \leq 0.01$), whereas no significant difference was found between the two asthma groups. Mean serum ECP was significantly increased at night in Group 2 ($p = 0.02$), but not in the other groups.

Correlations with circadian PEF-variation in asthmatic subjects

Bronchoalveolar lavage. Eosinophil and EG_2^+ cell numbers, mast cells, histamine levels and the other eicosanoid levels in the BAL fluid at 16.00 h, 04.00 h and (04.00 -

Table 3. Cellular aspects of peripheral blood. Medians with range.

	Group 1 Controls		Group 2 Asthma PEF-rhythm < 15%		Group 3 Asthma PEF-rhythm ≥ 15%	
	16.00	04.00	16.00	04.00	16.00	04.00 h
Total leukocytes x 10^9/L	7.8 (5.2-8.7)	8.1 (4.9-9.3)	7.1 (4.5-9.2)	7.3 (5.0-9.7)	7.2 (4.9-10.4)	7.6 (5.6-9.0)
Eosinophils x 10^6/L	88 (22-297)	198 (22-352)	264 (99-473)	308 (231-1002)[1]	276 (44-605)[2]	308 (121-1496)[2]
ECP (ng/ml)	13.6 (5.5-16.1)	10.9 (6.5-16.8)	16.1 (6.3-17.5)	16.9 (11.2-28.6)[1]	17.6 (16.4-60.3)[3]	17.8 (15.9-45.1)[2]

1 $p \leq 0.02$ compared with 16.00 h value within the group;
2 all asthmatic subjects, 16.00 and 04.00 h: $p \leq 0.02$;
3 $p \leq 0.03$ compared with 16.00 h values of Groups 1 and 2.
4 all asthmatic subjects, 16.00 h: $p < 0.001$ and 04.00 h: $p = 0.001$ compared with value of control group.

16.00) h were not significantly correlated with the circadian PEF-variation in asthmatic subjects, although a trend was found for higher PGD_2 levels at 16.00 h and higher EDN levels at 04.00 h with a bigger circadian PEF-rhythm ($r = 0.49$ and $r = 0.47$, respectively, $p = 0.06$).

Peripheral blood. ECP values at 16.00 h (also without outlier of 60.3) correlated significantly with the circadian PEF-variation in asthmatic subjects ($r = 0.61$, $p = 0.01$) (figure 3). Total eosinophil numbers both at 16.00 and 04.00 h, as well as (16.00 - 04.00 h) changes, were not significantly correlated with the circadian PEF-variation.

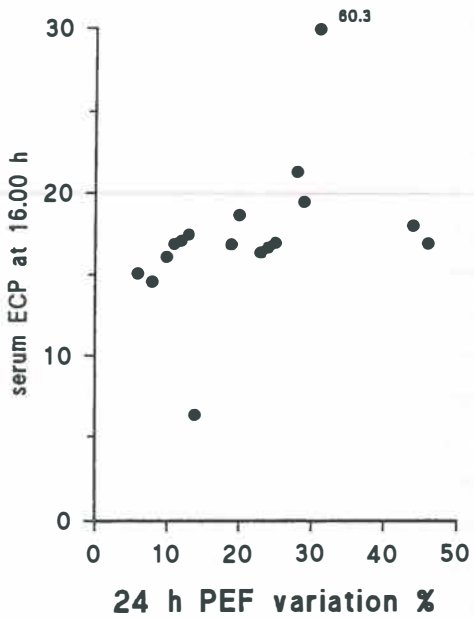


Figure 3. Relationship between serum ECP levels at 16.00 h and circadian PEF-variation in all asthmatic subjects ($r = 0.61$, $p = 0.01$).

Discussion

We investigated the presence of inflammatory cell numbers and their activation products in BAL fluid and PB and determined their relationship with the occurrence of increased nocturnal airways narrowing in asthma, as assessed by measurement of the circadian PEF-variation. Overall, higher eosinophil numbers and levels of inflammatory cell activation products were found in BAL fluid and PB from asthmatic subjects in comparison with control subjects. However, in asthmatic subjects with increased

circadian PEF-rhythm, no specific circadian change in inflammatory cells and the investigated mediators occurred. Most activation parameters at 16.00 and 04.00 h did not differentiate this group from asthmatic subjects without increased nocturnal airways narrowing. Interestingly, a number of eicosanoid levels in BAL fluid (PGD_2 and TxB_2), as well as serum ECP levels, were significantly higher in daytime in asthmatic subjects with increased circadian PEF-variation as compared to those without increased circadian PEF-variation. A significant correlation was found between serum ECP and circadian PEF-variation in asthmatic subjects. In addition, a trend was observed towards a correlation between 16.00 h BAL PGD_2 levels and circadian PEF-variation in asthmatic subjects, but no correlation whatsoever between the TxB_2 levels and the circadian PEF-variation existed. The results together suggest that increased airways narrowing at night is more likely to occur in asthmatic subjects with higher serum ECP and BAL PGD_2 levels.

So far, contradictory results have been reported on the recruitment of leukocytes to the airways at night in nocturnal asthma. Martin and colleagues (3) demonstrated significant increases in numbers of eosinophils and lymphocytes in BAL fluid, in association with increases in total white cell counts and neutrophil numbers. In contrast, Jarjour and colleagues (4) did not find changes in BAL cell number in their subjects with nocturnal asthma, which corresponds with our findings. A comparison of patient characteristics of these two studies and ours shows that the nocturnal asthmatic subjects in Martin's study generally had more severe asthma, with lower mean FEV_1 values and higher circadian PEF-variations at home. It is therefore possible that only in severe nocturnal airways narrowing an association can be found with pronounced infiltration of inflammatory cells into the airways, as recovered by BAL fluid. Furthermore, in both other studies most of the subjects used theophyllines at the time of investigation, whereas our subjects did not use theophylline, while maintenance therapy was withheld from four weeks before the start of the study. Although Martin reported that the mean serum theophylline concentrations at 16.00 and 04.00 h did not differ, it is not clear whether individual differences in serum concentrations were observed. Changes in theophylline levels are known to modulate PB eosinophil numbers (25) and it is not excluded that this may have affected airways leukocyte traffic in their study as well.

Contradictory results have also been reported on variations of PB eosinophil numbers and their activity between 04.00 and 16.00 h in nocturnal asthmatic subjects.

As the eosinophil shows a circadian chronobiological rhythm with the highest value at night around 2:00 A.M. in normal subjects (9), equal or increased numbers are likely to be found at night, depending on the time point of investigation. Eosinophil numbers in nocturnal asthmatic subjects were reported to be either normal (3) and increased (5, 6) at 4:00 A.M., or decreased (7). In our study, PB eosinophil numbers increased at night, without a difference between the 3 groups, whereas no variation in ECP levels between 04.00 and 16.00 h was found in the group with increased circadian PEF-variation. Fitzpatrick and coworkers (7) reported decreased eosinophil numbers, along with reduced serum ECP levels during the night and suggested these findings to be compatible with the involvement of a late asthmatic reaction in the pathogenesis of nocturnal asthma, although it cannot be excluded that the use of inhaled and oral corticosteroids in the latter study may have affected the results (6).

Interestingly, asthmatic subjects with increased circadian PEF-variation could be distinguished from asthmatic subjects without increased circadian PEF-variation by significantly higher levels of PGD_2 in BAL and ECP in serum at 16.00 h, suggesting that increased nocturnal airways obstruction occurs in asthmatic subjects with a pre-existing more severe degree of inflammation. PGD_2 is the major prostanoid release product of mast cells, although other cells in the airways - alveolar macrophages and eosinophils - can synthesize PGD_2 as well (26). Indirect evidence of a role for mast cell activation in nocturnal asthma has been suggested by findings that asthmatic subjects with increased nocturnal airways narrowing showed a higher airways hyperresponsiveness to adenosine 5'- monophosphate (1), which exerts its airways constrictive effect mainly by mast cell activation. Upon AMP provocation, PGD_2 levels in the BAL fluid have been reported to be increased (27). Inhalation of a non-contractile dose of PGD_2 has been shown to potentiate airways hyperresponsiveness to methacholine and histamine (28). Therefore, release of PGD_2 may play an important role in the increased airways hyperresponsiveness at night underlying increased nocturnal airways narrowing in asthma.

Increased serum ECP levels in asthmatic subjects with increased nocturnal airways narrowing were not accompanied with increased eosinophil activation in BAL fluid in our study. It is possible that measurement of eosinophil activation parameters in BAL fluid does not fully reflect eosinophil activation in the airways or only at a more severe stage of airway inflammation. Studies on biopsy specimens obtained from the bronchial wall may reveal additional information about the involvement of eosinophil

activation in increased nocturnal airways narrowing in asthma.

We did not find evidence for circadian fluctuations in cell number and mediators in the BAL fluid similar to the observed circadian fluctuations in PB samples. In healthy subjects the various PB cell types show different circadian rhythms, resulting in higher numbers of circulating lymphocytes and eosinophils and lower numbers of neutrophils during the night (9). These fluctuations were not found in the BAL cell numbers. BAL lymphocyte numbers did not change at night, whereas eosinophil numbers increased along with the neutrophil numbers in the control group and the asthma group without circadian PEF-rhythm. We did, however, not observe these changes in the asthma group with increased circadian PEF-rhythm. Possibly these cells are entrapped at night in increased numbers in the airway mucosa in asthmatic subjects with increased nocturnal airways narrowing. This assumption needs further investigation.

In conclusion, we found that increased nocturnal airways narrowing in asthmatic subjects is on the whole not associated with a circadian fluctuation in cell number and inflammatory mediators in PB and BAL fluid. In general, higher numbers of eosinophils and levels of cell activation products are found in BAL fluid and PB from asthmatic subjects as compared to control subjects. However, in asthmatic subjects with increased nocturnal airways obstruction most activation parameters did not differentiate them from asthmatic subjects without nocturnal airways obstruction. Interestingly, significantly higher levels of ECP in serum and PGD_2 in BAL fluid were present in asthmatic subjects with increased nocturnal airways obstruction. These findings suggest that increased nocturnal airways narrowing occurs in asthmatic subjects with ongoing increased non-specific cellular activation at daytime.

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Chapter 6

LYMPHOCYTE AND MACROPHAGE ACTIVATION IN BRONCHOALVEOLAR LAVAGE FLUID IN NOCTURNAL ASTHMA

Ytske Oosterhoff, Henk C. Hoogsteden, Bea Rutgers,
Henk F. Kauffman, Dirkje S. Postma

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Abstract

Background: Increased nocturnal airways narrowing is thought to occur as a consequence of an intensification of inflammatory processes at night. Lymphocyte and alveolar macrophage (AM) activation are thought to be associated with the clinical expression of asthma and may be important in the occurrence of nocturnal asthma as well.

Methods: The expression of CD25- and HLA-DR-receptors on bronchoalveolar lavage (BAL) and peripheral blood (PB) $CD4^+$ lymphocytes, as well as CD14-, IgG Fc- and CD11/CD18 leukocyte adhesion- receptors on AM in BAL and monocytes in PB were determined at 16.00 and 4.00 h by flow cytometry. Their relationship with the occurrence of nocturnal asthma was investigated in 8 non-atopic controls (Group 1) and 17 atopic asthmatic subjects, prospectively assigned to groups with a mean circadian peak expiratory flow (PEF)-variation $< 15\%$ (Group 2) and $\geq 15\%$ (Group 3).

Results: 1) The occurrence of increased circadian PEF-variation in asthmatic subjects was on the whole not associated with a day-night fluctuation in PB or BAL lymphocyte numbers and subsets, nor with day-night changes in BAL AM or PB monocyte and receptor expression. The only exception was the presence of a higher day-night change in the proportion of *HLA-DR-expressing $CD4^+$ lymphocytes* in the BAL fluid along with an increasing circadian PEF-rhythm in asthmatic subjects ($r = 0.68$, $p = 0.03$).

2) A lower number of *BAL $CD4^+$ lymphocytes* at daytime was significantly related to a higher circadian PEF-variation in asthmatic subjects ($r = -0.66$, $p = 0.01$).

3) Asthmatics in Group 3 showed a higher expression of CD11b on *BAL AM* at 16.00 h as compared to both other groups ($p = 0.05$), and a significantly positive correlation was found for this parameter with the circadian PEF-variation of all asthmatic subjects ($r = 0.72$, $p = 0.03$).

Conclusions: Increased nocturnal airways obstruction in asthma is not associated with an increased influx of lymphocytes and macrophages in BAL-fluid at night, nor with marked activation of these cells. Daytime presence of enhanced AM CD11b expression may predispose for the occurrence of nocturnal asthma.

Introduction

Nocturnal and early morning dyspnea are common symptoms in asthma. The dyspnea is the result of an increased airways narrowing at night, as reflected by larger swings in airway diameter than observed in many other asthmatic patients and in healthy

individuals (1). The increased nocturnal airways narrowing is thought to occur as a consequence of an intensification of inflammatory processes in the airways of subjects with nocturnal asthma, as has been suggested by an increase in (indirect) airways hyperresponsiveness at night (2).

Inflammatory conditions may be associated with an increased influx of cells into the airway wall, along with marked shifts in cell populations in the bronchoalveolar lavage (BAL) fluid. So far, however, conflicting results have been reported on nocturnal increases in cell numbers and morphology, as analysed in BAL fluid from subjects with nocturnal asthma (3-5). Information on the contribution of inflammation may more appropriately be obtained by studying cell activation. In this study, the role of lymphocyte and AM activation in the occurrence of nocturnal asthma was investigated by determination of the expression of specific cell surface receptors associated with cell activation. Both lymphocyte (6, 7) and alveolar macrophage (AM) (8) activation are thought to be associated with the clinical manifestation of asthma. The number of activated $CD4^+$ lymphocytes in BAL has been reported to be related with the severity of asthma (6) and $CD4^+$ lymphocytes could be detected in peripheral blood (PB) during acute asthma attacks to a degree inversely correlating with clinical improvement (7). Macrophage activation has been reported in asthma as well, as reflected by an influx of monocyte-like macrophages into the submucosa and increased release of inflammatory products by AM (8). We therefore have set out to investigate whether increased numbers and activation of BAL and PB lymphocytes, AM in BAL-fluid and monocytes in PB were related to the occurrence of nocturnal asthma. Determinations were performed at 16.00 and 04.00 h, both in healthy subjects and atopic asthmatic subjects, who were prospectively assigned to a group with or without an increased circadian peak expiratory flow (PEF)-variation.

Materials and methods

Study design

Eight healthy and 17 asthmatic subjects (18-45 years) participated in the study. The week before the study, PEF-values were recorded with a Wright mini peak flow meter during three days at 08.00, 12.00, 16.00, 20.00, 24.00 and 04.00 h. Bronchodilators ($ß_2$-agonists or ipratropiumbromide) were withheld during these days. The best of three PEF-maneuvers was recorded. The mean circadian PEF-variation of the three days was defined as: (highest - lowest 24 h value)/mean 24 h value. Subjects were prospectively

selected in three groups: *Group 1* consisted of the 8 healthy volunteers, *Group 2* the 7 asthmatic subjects with a mean circadian PEF-variation < 15 % and *Group 3* the 10 asthmatic subjects with a mean circadian PEF-variation $\geq$ 15 %. Forced expiratory volume in one second (FEV_1) and airways hyperresponsiveness to methacholine, defined as the provocative concentration of methacholinebromide that caused a 20 % fall in FEV_1 (PC_{20}), were assessed within 5 days before the first bronchoscopy. FEV_1 was measured with a calibrated water-sealed spirometer according to standardized guidelines (9). Airways responsiveness to doubling concentrations of 0.03 to 9.8 mg/ml methacholinebromide (Sigma Chemical co., ST. Louis, MO) was measured using a 2-minute tidal breathing method adapted from Cockcroft and coworkers, as described previously (2).

Thirty minutes before each bronchoscopy, FEV_1 measurements were performed and blood samples were taken. Subjects rested during 15 minutes before the lung function measurements in daytime. At night, subjects were admitted to the hospital at 22.00 h and were awakened 15 minutes before the measurements. Bronchodilator therapy was withheld 8 h before the pulmonary function tests. Bronchoscopies were performed in randomized order at 16.00 h and 04.00 h on two different days, seperated by at least seven days. Blood and BAL samples were processed immediately in the laboratory. Phenotypic characteristics of blood and BAL cells were analysed by flow cytometry. As a consequence of a limited yield of BAL cells, analysis of activated $CD4^+$ lymphocytes and AM could not be performed in all experiments.

Subjects

The study was approved by the ethical committee of our hospital, and written informed consent was given by each subject.

Healthy volunteers had: 1. no history of lung disease or allergy; 2. no atopy: negative intracutaneous tests against 18 common aeroallergens, expressed as histamine equivalent wheal size (HEWS) < 0.7 (Diephuis Laboratories, Groningen, the Netherlands); 3. no airways response upon provocation with methacholine: < 10% fall in FEV_1 after inhalation of methacholinebromide in a concentration of 9.8 mg/ml); 4. no upper respiratory infections within 1 month before the study and no concomitant diseases.

Asthmatic subjects had: 1. a history of episodic dyspnea or wheezing consistent with the clinical diagnosis of asthma (10); 2. atopy: positive intracutaneous tests

against housedust mite or two other of 18 tested common aeroallergens (HEWS $\geq$ 0.7); 3. FEV_1 > 1.50 l and $\geq$ 70% predicted; post-bronchodilator FEV_1 (salbutamol 400 μg using a spacehaler) $\geq$ 80 % predicted; 4. PC_{20} methacholine $\leq$ 9.8 mg/ml; 5. no use of oral corticosteroids within 2 months, inhaled corticosteroids within four weeks and cromoglycates within two weeks respectively before the study; 6. no upper respiratory infections within 1 month before the study and no other concomitant diseases.

Bronchoscopy procedure

Fiberoptic bronchoscopy was undertaken according to guidelines of the American Thoracic Society (11). Premedication consisted of intramuscular injection of 0.5 mg atropine and 200 μg salbutamol directly before the procedure. Lidocaine 4% was administered into the upper airways and bronchial tree. Bronchoscopy was performed using an Olympus B1 IT10 flexible fiberoptic bronchoscope (Olympus Optical, Tokyo, Japan). Ten aliquots of 20 ml sterile phosphate-buffered saline (PBS) of 37 °C were instilled into the lateral segment of the right middle lobe and recovered by gentle suction (-40 cm H_2O) after each aliquot. The BAL fluid was collected in polypropylene aliquots, immediately placed in ice.

Laboratory techniques

Processing of BAL fluid. The lavage fluid was filtered through a 100 μm pore filter of a venous infusion system (Curapharm, Medica B.V., Hospital Supplies, the Netherlands) to remove mucus, and centrifuged at 400 g at 4° C for 5 minutes. The cell pellets were resuspended in 1 ml PBS supplemented with 0.1% glucose (PBS/BSA 0.5%). Total leukocyte numbers in BAL cell suspensions were counted in a Coulter Counter (Model S-plus VI; Coulter Electronics, Hialeah, USA) and viability was assessed by cellular exclusion of trypan blue. BAL cytospin preparations were made from 100 μl fractions of the BAL cell suspension, diluted to a volume containing 0.25 x 10^6 cells/ml in PBS supplemented with 0.5% heat-inactivated bovine serum albumin (PBS/BSA 0.5%), using a Shandon cytospin-2 centrifuge (Shandon Inc, Pittsburgh, USA). After staining with May-Grünwald-Giemsa, cell differential counts were performed on two slides. The avarage of 600 cells on BAL cytospin slides was taken as the definite differential cell count. For immunofluorescence staining, 250 μl BAL cell suspension (1.2 x 10^6 cells/ml) was centrifuged at 500 g at 10° C for 5 minutes and subsequently

incubated at 4° C for 30 minutes with the appropriate dilutions of McAb and 5 % human AB-serum (Red Cross Bloodbank, Groningen, the Netherlands), in order to prevent non-specific binding of antibody. After incubation, the cells were washed twice with PBS/BSA 0.5% and centrifugation at 1000 g at 10° C for 2 minutes. Subsequently, samples with unconjugated McAb were incubated at 4° C for 30 minutes with goat anti-mouse fluorescein isothiocyanate (GAM FITC) solution and 5% AB serum. After washing and centrifugation, 150 μl formaldehyd 0.05% (Merck, Darmstadt, Germany) was added for fixation of the cells. The cells were stored in the dark at 4 °C until flow cytometric analysis.

Processing of blood. Total leukocyte numbers in EDTA-anticoagulated blood were counted in a Coulter Counter. Viability was assessed by cellular exclusion of trypan blue. Cell differentiation was asessed on two slides after staining of blood smears with May-Grünwald-Giemsa. The avarage of 200 cell counts was taken as the definite differential cell count. Imunofluorescence staining was performed as described previously (12).

Flow cytometry. Flow cytometric measurements were performed with a Facs 440 (Becton Dickinson). In blood, a minimum of 20,000 events was collected for each sample. Analysis was performed using software (Lysys; Becton-Dickinson). The blood lymphocytes and monocytes were seperately gated according to their forward and sideward scatter, after verifying the purity using CD45 and CD14 antibodies. For analysis of BAL AM, a minimum of 10,000 events was collected for each sample. BAL lymphocytes were counted separately after setting an electronic gate around the lymphocytes first. To subtype the PB and BAL lymphocytes, a double staining procedure was used. The cells were incubated with appropriate dilutions of McAb conjugated with a FITC or phycoerythrin (PE) fluorochrome against CD3, CD4 and CD8. Anti-CD25 and anti-HLA-DR in combination with anti-CD4 were used as indices of $CD4^+$ lymphocyte activation (table 1). FITC and PE conjugated mouse immunoglobulins of matched isotypes (IgG_1, IgG_{2a}) were used as negative controls. The percentage of positive cells expressing the surface marker was assessed.

To subtype the PB monocytes and BAL AM, the expression of the CD11/CD18 leukocyte integrin subunits (CD11a, CD11b, CD11c, CD18), IgG-Fc receptors (CD16, CD32w, CD64) and the monocyte marker CD14 were investigated (table 1). The

Table 1. Survey of monoclonal antibodies

Code	Receptor	McAb
CD3	T3 antigen	Leu-4
CD4	T4 antigen	Leu-3a
CD8	T8 antigen	Leu-2a
CD25	α-chain IL-2 receptor	2A3
	HLA-DR	L243
CD11a	LFA-1 antigen	LFA-1α
CD11b	C3bi receptor	Leu-15
CD11c	p150/95 antigen	Leu-M5
CD18	ß-chain CD11 antigen	LFA-1ß
CD64	IgG-FcR I	32.2
CD32w	IgG-FcR II	IV.3
CD16	IgG-FcR III	Leu-11b
CD14	monocytic antigen	UCHM1

expression of cell surface receptors by McAb was assessed as the mean fluorescence intensity (MFI) in arbitrary units transformed to a linear scale from $\log_{10}$ channel number of mean fluorescence. For proper comparison of the fluorescence intensity values from the different experiments assessed during one year, a standard set of FITC calibrated microbeads (Becton Dickinson) was taken along during each experiment. After transformation via the MFI - FITC equivalents curve for each experiment, the fluorescence intensity, expressed as $\log_{10}$ FITC equivalents x 10^3/mol soluble microbeads, was calculated by subtraction of the proper control isotype values from the values of the investigated McAb.

Data analysis

Values are presented as medians with ranges. After verifying the normal distribution, spirometry changes between groups were analysed with the Student t-test for unpaired observation. Number of positive skin tests, nocturnal asthma-score, BAL and PB parameters were not normally distributed and were therefore analysed with the Mann-Whitney U test comparing data between groups, and Wilcoxon's matched signed rank tests for within-group analysis. Correlations between the cell parameters and the circadian PEF-variations were made using Spearman's rank correlation tests. All analyses were performed with the SPSS/PC$^+$ V 4.01 software package (SPSS Inc., Chicago, USA). P values ≤ 0.05 were considered statistically significant.

Results

Subjects

Subject characteristics of the three groups are listed in table 2. Asthmatic subjects in Group 3 scored significantly higher on awakening due to nocturnal dyspnea ($p = 0.03$)

and their geometric mean PC_{20} methacholine was significantly lower as compared to the asthmatic subjects in Group 2 ($p = 0.02$). Log_2 PC_{20} methacholine values of all asthmatic subjects were significantly correlated to the circadian PEF-variation ($r = -0.64$, $p = 0.005$). Mean FEV_1% predicted at 16.00 h was significantly lower in both asthma groups compared with the healthy control group ($p < 0.05$), but not between both asthma groups. In Group 3 mean FEV_1 fell significantly during night as compared with the 16.00 h value ($p = 0.005$).

Table 2. Subject characteristics. Medians with range

	Group 1	Group 2	Group 3
Number	8	7	10
Circadian PEF-rhythm %	9 (4-11)	11 (6-14)	27 (19-46)
Age, yrs	25 (20-44)	22 (18-40)	24 (20-41)
Gender, M/F	4/4	5/2	3/7
Smokers	0/8	0/7	2/10
Positive skin tests	0	6 (6- 7)	6 (2- 8)
PC_{20} Methacholine (mg/ml)	> 9.8	1.33 (0.34-1.88)	0.48 (0.04-0.96)[1]
NA-score	0	0 (0- 1)	1 (0- 2)[2]
FEV_1% pred. 16.00 h	103 (88-125)	94 (80-105)	89 (68-114)[3]
FEV_1% pred. 04.00 h	103 (87-122)	90 (70- 97)	75 (47-104)[4]

1 $p = 0.02$ compared with Group 2 (geometric mean value);
2 Awakening because of nocturnal dyspnea in previous two weeks: 0 = never, 1 = sometimes, 2 = every night; $p = 0.03$ compared with Group 2;
3 $p < 0.05$ compared with control group;
4 $p = 0.005$ compared with daytime value.

BAL parameters

All subjects tolerated the bronchoscopies well. At discharge three hours after the bronchosopy procedure, PEF measurements were comparable to the values before bronchoscopy. Results of BAL parameters in Group 3 are based on data from 9 subjects, as one subject refused a second bronchoscopy. The recovery of BAL fluid, total leukocyte and AM numbers were not significantly different among the various groups at the different time points, nor were variations between 16.00 and 04.00 h within the groups observed (table 3).

Table 3. BAL and PB cellular data.

	Group 1 Controls		Group 2 Asthma PEF-rhythm < 15%		Group 3 Asthma PEF-rhythm ≥ 15%	
	16.00	04.00	16.00	04.00	16.00	04.00 h
BAL leukocyte numbers and lymphocyte subpopulations x 10^3/ml						
Recovery %	73 (53 - 83)	70 (61 - 82)	77 (13 - 86)	70 (21 - 87)	62 (51 -79)	58 (10 - 86)
Total leukocytes	56.7 (29.8-124)	72.3 (28.8-137)	52.1 (18.2-112)	98.2 (28.5-159)	67.4 (7.5-413)	92.1 (12.3-276)
AM	54.6 (29.0-117)	66.4 (25.2-133)	49.3 (15.8- 99)	89.5 (26.2-154)	66.9 (6.7-401)	86.6 (12.0-238)
Lymphocytes	1.0 (0.2- 6.2)	1.5 (0.3- 4.5)	1.3 (0.7- 5.7)	2.4 (0.7- 6.4)	0.8 (0 - 3.3)	0.5 (0 - 2.6)[1]
$CD3^+$	0.9 (0.2- 5.5)	1.3 (0.2- 4.3)	2.3 (1.1-4.8)	2.8 (1.9- 5.2)[2]	0.8 (0 - 1.7)	0.5 (0 - 1.6)[1]
$CD4^+$	0.6 (0.1- 3.5)	0.9 (0.2- 2.9)	0.7 (0.4-2.5)	1.2 (0.3- 3.2)	0.4 (0 - 1.2)	0.3 (0 - 1.4)
$CD8^+$	0.3 (0.1- 2.2)	0.4 (0.1- 1.3)	0.6 (0.2-2.2)	0.8 (0.2- 2.1)	0.3 (0 - 2.8)	0.3 (0 - 1.1)
Blood leukocyte numbers and lymphocyte subpopulations x 10^6/ml						
Total leukocytes	7.8 (5.2- 8.7)	8.1 (4.9- 9.3)	7.1 (4.5-9.2)	7.3 (5.0-9.7)	7.2 (4.9 -10.4)	7.6 (5.6- 9.0)
Monocytes	0.5 (0.3- 0.8)	0.6 (0.2- 0.9)	0.3 (0.1-0.6)	0.4 (0.2-0.5)	0.4 (0.2 - 1.0)	0.5 (0.2 -0.8)
Lymphocytes	1.9 (1.4- 2.7)	2.8 (2.1- 3.3)[3]	2.1 (1.2-3.1)	2.5 (1.9-3.8)	1.9 (1.2 - 3.8)	2.6 (1.8-4.2)[3]
$CD3^+$	1.4 (0.8- 2.0)	2.2 (1.3- 2.8)[3]	1.5 (0.9-2.2)	1.9 (1.4-3.0)[3]	1.4 (0.9 - 2.9)	2.0 (1.4-3.5)[3]
$CD4^+$	0.8 (0.4- 1.2)	1.3 (0.9- 1.9)[3]	0.9 (0.4-1.4)	1.1 (0.7-1.9)	0.9 (0.4 - 1.8)	1.2 (0.9-2.3)[3,4]
$CD8^+$	0.6 (0.4- 0.7)	0.8 (0.5- 0.8)[3]	0.6 (0.4-0.8)	0.8 (0.5-1.1)	0.6 (0.4 - 1.1)	0.8 (0.4-1.1)[4]

1 $p \leq 0.04$ compared with 04.00 h value of Group 2;
2 $p \leq 0.03$ compared with 04.00 h value of control group;
3 $p \leq 0.03$ compared with 167.00 h values within the group;
4 groups 2 + 3: $p \leq 0.05$ compared with mean 16.00 h value.

BAL lymphocytes and subpopulations. Total, $CD3^+$ and $CD4^+$ lymphocyte numbers in Group 3 were lower as compared to the other groups at both time points, reaching significance between the two asthma groups at 04.00 h for total and $CD3^+$ lymphocytes ($p \leq 0.04$) (table 3). The number of $CD8^+$ lymphocytes was not significantly different between the three groups. Total and subset lymphocyte numbers showed no significant variations between 16.00 and 04.00 h within the three groups.

Total lymphocyte numbers of all asthmatic subjects at both time points were inversely correlated with circadian PEF-rhythm, being significant at 04.00 h ($r = -0.52$, $p = 0.05$). $CD4^+$ subsets also showed negative correlations with circadian PEF-rhythm, this was significant at 16.00 h ($r = -0.66$, $p = 0.01$; figure 1).

Activation of BAL $CD4^+$ lymphocytes. The percentage of HLA-DR^+ $CD4^+$ lymphocytes was not significantly different among the three groups at both time points (figure 2). A significant variation in percentage of HLA-DR^+ $CD4^+$ lymphocytes between 16.00 and 04.00 h was found in Group 3 ($p = 0.05$), with an opposite trend in Group 2 ($p = 0.07$). Besides, a significant correlation existed between the (16.00 - 04.00 h) percentage of HLA-DR^+ $CD4^+$ lymphocytes of all asthmatic subjects and the circadian PEF-variation ($r = 0.68$, $p = 0.03$; figure 3).

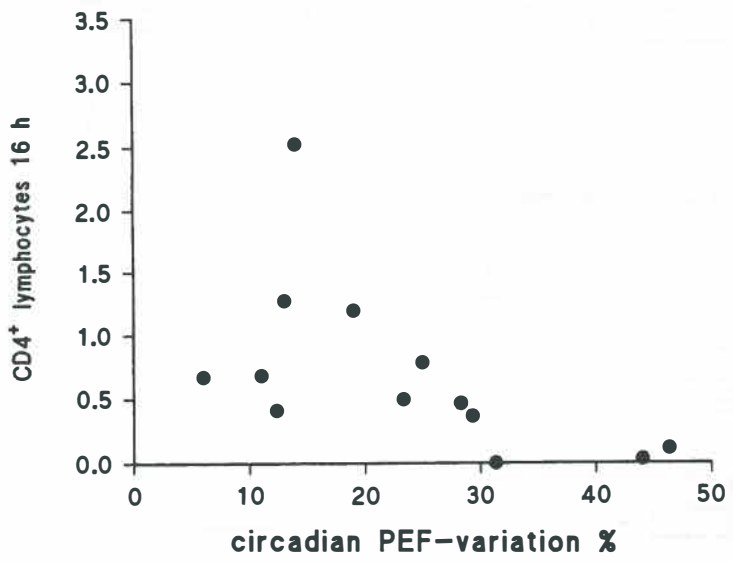

Figure 1. Relationship between BAL $CD4^+$ lymphocyte numbers (x 10^3/ml) at 16.00 h and the circadian PEF-variation in asthmatic subjects ($r = -0.66$, $p = 0.01$).

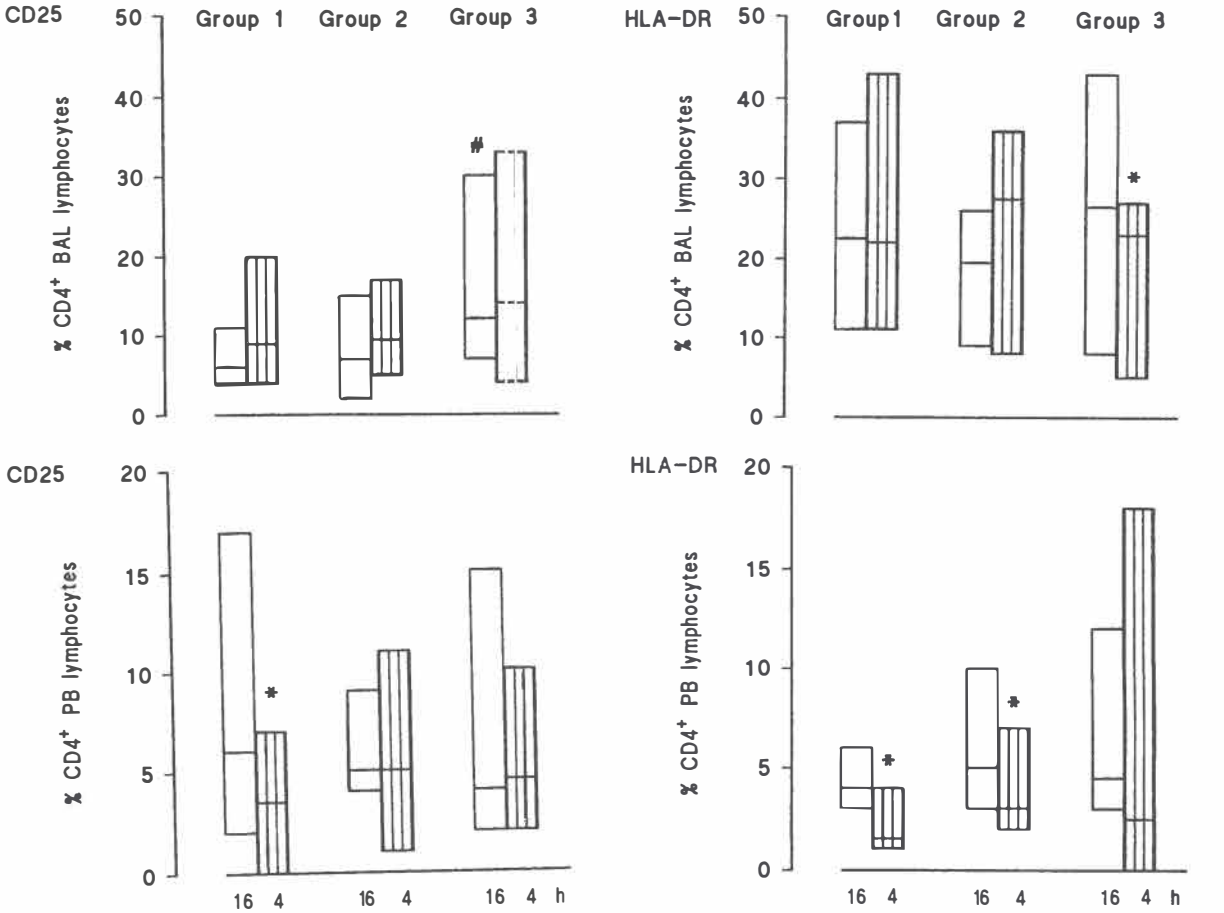

Figure 2.
Percentage of BAL (upper figure) and blood (lower figure) $CD4^+$ lymphocytes expressing HLA-DR and CD25 in control subjects (*Group 1*), asthmatic subjects with circadian PEF-variation < 15% (*Group 2*) and ≥ 15% (*Group 3*) at 16 h (open bars) and 04 h (striped bars), expressed as median value with ranges. $^*p \leq 0.05$ compared with 16 h value, $^\#p = 0.03$ compared with 16 h value of control group.

The percentage of $CD25^+$ $CD4^+$ lymphocytes in Group 3 was higher as compared to the other groups, reaching significance with the control group at 16.00 h ($p = 0.03$) (figure 2). However, no correlation was found between the percentage of $CD25^+$ $CD4^+$ lymphocytes of asthmatic subjects and the circadian PEF-variation. Variations in percentage of $CD25^+$ $CD4^+$ lymphocytes between 16.00 and 04.00 h within the groups were neither observed.

BAL AM receptor expression. The expression of CD11b on AM at 16.00 h was the only significant difference between Group 3 and both other groups ($p = 0.05$; table 4). The expression of CD11b on AM of all asthmatic subjects at 16.00 h was significantly correlated with the circadian PEF-variation ($r = 0.72$, $p = 0.03$; figure 4). No variations in receptor expression on AM between 16.00 and 04.00 h within the groups were found.

Peripheral blood parameters

Total leukocyte and monocyte numbers were not significantly different among the various groups at the different time points, nor variations between 16.00 and 04.00 h within the groups were observed (table 3).

PB lymphocyte and subpopulations. No significant differences in total number of lymphocytes and subsets were found between the control and both asthma groups at both time points (table 3). Lymphocyte numbers and their subsets increased significantly at 04.00 h as compared with 16.00 h in control subjects and all asthmatic subjects ($p \leq 0.03$).

PB activated $CD4^+$ lymphocytes. No significant differences in percentage of $HLA\text{-}DR^+$ and $CD25^+$ $CD4^+$ lymphocytes were found between the groups at both time points (figure 2). The percentage of $HLA\text{-}DR^+$ $CD4^+$ lymphocytes decreased significantly at 04.00 h as compared to 16.00 h in the control group ($p = 0.02$) and both asthma groups ($p = 0.04$). The percentage of $CD25^+$ $CD4^+$ lymphocytes also decreased significantly in all normal subjects ($p = 0.03$; figure 2), but not in both asthma groups. However, the (16.00 - 04.00 h) variation in percentage of $CD25^+$ $CD4^+$ lymphocytes of all asthmatic subejcts was not significantly correlated with the circadian PEF-variation.

PB monocyte receptor expression. The expression of CD16 on monocytes at 16.00 h was significantly lower in Group 3 as compared to both other groups ($p \leq 0.05$; MFI median [range], Group 3: 12 [0-43], Group 2: 29 [0-37] and Group 1: 29 [5-42]).

Table 4. Receptor expression on BAL AM. Medians with range[1]

	Group 1 Controls		Group 2 Asthma PEF-rhythm < 15%		Group 3 Asthma PEF-rhythm ≥ 15%	
	16.00	04.00	16.00	04.00	16.00	04.00 h
CD11a	57 (29- 78)	64 (13- 95)	55 (51- 64)	53 (22- 83)	62 (28-109)	33 (21- 65)
CD11b	35 (26- 42)	39 (14- 67)	34 (25- 42)	42 (30- 61)	**45 (39- 66)**[2]	33 (10- 43)
CD11c	66 (50- 81)	71 (27- 87)	62 (44- 78)	74 (55- 85)	64 (38-106)	42 (22- 77)
CD18	50 (21- 95)	42 (0- 69)	36 (28- 55)	29 (0- 69)	38 (13-115)	28 (0- 64)
CD64	33 (8- 67)	35 (17- 56)	34 (21- 42)	36 (31- 48)	34 (22- 67)	17 (5- 53)
CD32w	81 (61- 95)	80 (43-107)	74 (64- 80)	69 (60- 88)	73 (56-118)	58 (34- 87)
CD16	51 (11- 94)	41 (15- 75)	36 (16- 42)	39 (29- 65)	43 (28- 98)	25 (3- 56)
CD14	8 (0- 12)	8 (0- 26)	4 (0- 7)	11 (2- 13)	5 (4- 22)	0 (0- 6)

1 Expressed as $\log_{10}$ FITC equivalents x 10^3/mol soluble microbeads (x 10^2);
2 $p \leq 0.05$ compared with 16 h values of Groups 1 and 2.

However, the CD16-expression on monocytes of all asthmatic subjects at this time point was not significantly correlated with the circadian PEF-variation. The expression of the other investigated phenotypes on monocytes were not significantly different between the three groups, neither were variations in phenotype expression on monocytes between 16.00 and 04.00 h within the different groups.

Discussion

In this study it was investigated whether increased numbers and activation of BAL and PB lymphocytes and AM in BAL-fluid were related to the occurrence of increased nocturnal airways obstruction in asthma.

We found that the occurrence of increased circadian PEF-variation in asthmatic subjects is on the whole not associated with day-night changes in number and activation state of PB and BAL lymphocytes or AM in BAL-fluid. The only exception is the presence of a higher day-night change in the proportion of HLA-DR-expressing $CD4^+$ lymphocytes in the BAL fluid along with a higher circadian PEF-rhythm in asthmatic subjects.

Unexpectedly, we found lower numbers of BAL total, $CD3^+$ and $CD4^+$, but not $CD8^+$ lymphocytes, in asthmatic subjects with increased circadian PEF-rhythm. Significantly negative correlations were found between total lymphocyte numbers of all asthmatic subjects at 04.00 h and circadian PEF-variation, as well as $CD4^+$ lymphocyte numbers at 16.00 h and circadian PEF-variation. In contrast to our findings, no differences in lymphocyte numbers were reported between the nocturnal and non-nocturnal asthma or control groups in previous studies on nocturnal asthma (3-5). Although the reason for this discrepancy is not completely clear, two major differences are manifest comparing the patient characteristics of the different studies. The participants with nocturnal asthma in the studies from Martin (3) and Mackay (5) et al had generally more severe asthma, with lower FEV_1 values and higher circadian PEF-values at home. It is therefore possible that only in more severe nocturnal airways narrowing a pronounced infiltration of inflammatory cells occurs into the airways, as recovered by BAL fluid. Furthermore, in these studies most of the subjects used theophyllines (3,4) or inhaled steroids (5) at the time of investigation, whereas our subjects did not use maintenance therapy from four weeks before the start of the study. Anti-inflammatory treatment with inhaled corticosteroids has been reported to result in a significant increase in BAL lymphocyte numbers (13). Theophylline treatment

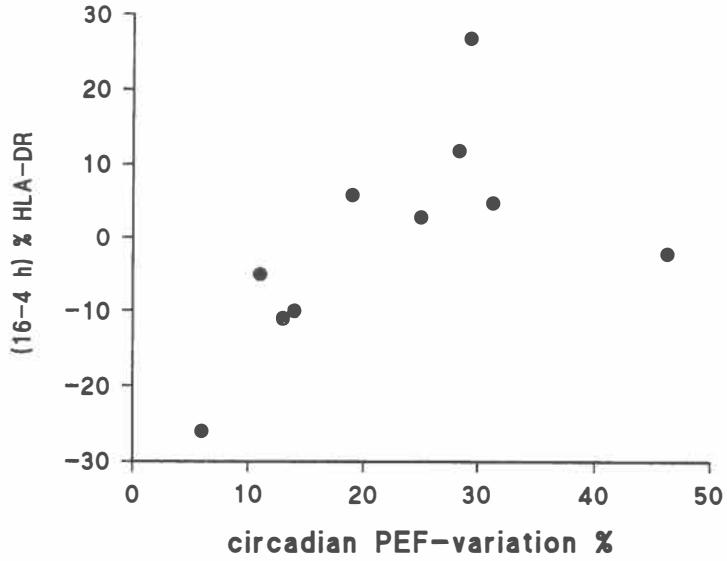

Figure 3. Relationship between the (16.00 - 04.00 h) percentage of $CD4^+$ lymphocytes expressing HLA-DR and the circadian PEF-variation in asthmatic subjects ($r = 0.68$, $p = 0.03$).

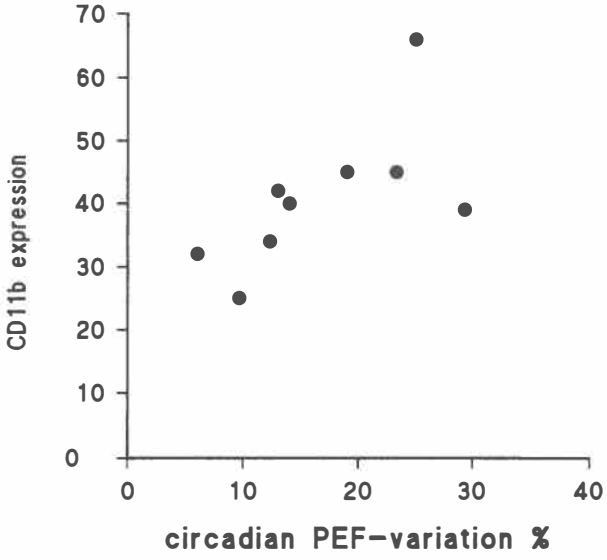

Figure 4. Relationship between the expression of CD11b on AM ($MFI * 10^2$) at 16.00 h and the circadian PEF-variation in asthmatic subjects ($r = 0.72$, $p = 0.03$).

has been shown to increase blood $CD8^+$ lymphocyte numbers (14) and it cannot be excluded that this may have affected airways lymphocyte traffic in previous studies as well.

The significance of lower BAL $CD4^+$ lymphocyte numbers in asthmatic subjects with increased circadian PEF-rhythm is not clear. It has been shown that after anti-inflammatory treatment T-lymphocyte numbers in the bronchial submucosa decreased (15), whereas BAL-lymphocyte numbers increased (13). From these observations it can be hypothesized that the lower BAL $CD4^+$ lymphocyte numbers are a manifestation of inflammation in the airways. Possibly these cells are entrapped in increased numbers in the airway mucosa in subjects with nocturnal asthma, where they become activated themselves or may activate other inflammatory cells. More research is needed on lymphocyte activation in the bronchial submucosa in association with the occurrence of nocturnal asthma.

CD11b expression on AM was the only activation marker that was found to be significantly higher in asthmatic subjects with increased circadian PEF-rhythm as compared to the other groups and it was positively correlated with the circadian PEF-rhythm in asthmatic subjects as well. The expression of the other investigated activation markers on AM may have been found not significantly different as a consequence of too small group sizes. CD11b is a complement receptor (CR3) that is constitutively expressed on many cell types. CR3 expression on AM is constitutively expressed in lower levels than on PB monocytes (16, 17), indicating that the expression of this receptor is down-regulated during migration of blood monocytes to the alveoli. Enhanced expression of CR3 on AM may therefore reflect local activation by T lymphocyte derived macrophage activating cytokines (18). The expression of complement receptors has been found to be elevated on circulating granulocytes of asthmatic patients after an experimentally provoked asthmatic reaction (19) and can be increased *in vitro* by several factors, among which the cytokines IL-3, IL-5 and GM-CSF (20). IL-4 has been shown to induce upregulation of CD11b expression on human macrophages *in vitro* (21). It can be assumed that increased expression of the CR3 receptor on AM is a consequence of previous exposure to cytokines in vivo, reflecting enhancement of ongoing inflammation in the asthmatic airways.

In conlusion, the occurrence of increased nocturnal airways obstruction in asthma is on the whole not associated with day-night changes in number and activation state of PB and BAL lymphocytes or AM in BAL-fluid. The only exception is the

presence of relatively less HLA-DR-expressing $CD4^+$ lymphocytes in the BAL fluid at night along with a higher circadian PEF-rhythm in asthmatic subjects. A lower number of BAL $CD4^+$ lymphocytes in association with a higher circadian PEF-rhythm in asthmatic subjects may reflect a lagging behind of these cells in the bronchial wall and needs further research on cellular activity in the bronchial submucosa in association with the occurrence of increased nocturnal airways obstruction. The finding of a higher CD11b expression on BAL AM at daytime in asthmatic subjects with increased circadian PEF-rhythm suggests that increased nocturnal airways narrowing is more likely to occur in asthmatic subjects with daytime existence of enhanced AM activation.

Acknowledgements

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Chapter 7

EOSINOPHIL AND LYMPHOCYTE MARKERS IN PERIPHERAL BLOOD, BRONCHIAL SUBMUCOSA AND BRONCHOALVEOLAR LAVAGE FLUID AT 04:00 AND 16:00 HOURS IN HEALTHY SUBJECTS AND ATOPIC ASTHMATICS

Roel M. Aleva, Ytske Oosterhoff, Mieke Smith, Dirkje S. Postma,
Jan Kraan, Wim Timens

Submitted

Abstract

Patients with asthma frequently have a nocturnal increase in symptoms, which has been associated with increased airways inflammation at night. The aim of the present study was to investigate proportional changes in number and activation state of lymphocyte subsets and eosinophilic granulocytes between day and night in patients with atopic asthma and in healthy subjects. As we supposed that these cell types move from peripheral blood into the airways, we investigated peripheral blood, bronchial biopsy and bronchoalveolar lavage (BAL) fluid samples obtained at 16:00 and 04:00 hours.

Cells from BAL-fluid and snap-frozen bronchial mucosa sections from non-smoking, age-matched healthy subjects (n = 8) and atopic asthmatics (n = 6) were stained for T-lymphocytes and subsets, and activated (EG2+) eosinophils. Numbers of submucosal CD4+ cells were higher in asthmatics than in controls at both points of time. A higher percentage CD4+ cells in peripheral blood was associated with more CD4+ cells in submucosal biopsies at 04:00 hours, and a higher number of submucosal CD4+ cells was associated with a lower % of BAL CD4+ cells in asthmatics. Asthmatics had higher numbers of eosinophils in peripheral blood than healthy individuals in association with both higher numbers of EG2+ cells in the bronchial submucosa and in BAL fluid at both points of time.

The results of this study together suggest that CD4+ cells are attracted from peripheral blood to the bronchial submucosa where they stay localized at 04:00 hours, whereas eosinophil recruitment from the peripheral blood to the bronchial submucosa occurs at both time points, where they move on into the airway lumen. More studies are required to determine the exact role of inflammation in nocturnal asthma.

Introduction

Inflammatory events in the bronchial mucosa are thought to underlie increased airways responsiveness in patients with atopic asthma, involving both eosinophils and lymphocytes (1,2). Patients with asthma frequently have a nocturnal increase in symptoms, which occurs simultaneously with an increase in circulating eosinophils (3) and an increase in airways hyperresponsiveness. Both endogenous factors i.e. circulatory variations in cortisol and adrenaline (4,5), and exogenous factors such as exposure to house dust mite in mattresses and beddings at night have been thought to be related to the exaggerated swing in airway diameter (6). The central hypothesis in

our study is that an influx of activated inflammatory cells in the bronchial submucosa at the time of the nocturnal airways narrowing due to both mechanisms is essential to the pathogenesis of nocturnal asthma.

The aim of the present study was to investigate proportional changes in number and activation state of lymphocyte subsets and eosinophilic granulocytes between day and night in patients with atopic asthma and in healthy subjects. As we suppose that these cell types move from peripheral blood to the airways, we investigated peripheral blood, bronchial biopsy and broncho-alveolar lavage (BAL) fluid samples obtained at 16:00 and 04:00 hours.

Material and methods

Subjects

Eight healthy control subjects (20-44 years) and 6 atopic asthmatic subjects (19-41 years), all non-smokers, enrolled in the study. Asthmatics had a positive skintest for house dust mite or two other common aeroallergens (HEWS $\geq$0.7) (7), their mean FEV_1 (% pred) was 92.2 (range 80 to 100), whereas the geometric mean fall in FEV_1 $\geq$ 20% after challenge with doubling concentrations of methacholine (PC_{20}), using a two minute tidal breathing method (8), was 1.01 mg/ml (range 0.36 - 1.68). Two asthmatic subjects had increased nocturnal airways obstruction as measured by a circadian PEF-variation ([$PEF_{highest}$-PEF_{lowest}/PEF_{mean} in 24 h) $\geq$ 15%, measuring PEF-rates every 4 hours during 3 consecutive days. Regular anti-asthma therapy, consisting of inhaled corticosteroids and cromolyns, were withheld within 4 and 2 weeks respectively before the study. Inhaled β_2-agonists or ipratropiumbromide were allowed.

Healthy volunteers had no history of allergy and respiratory disease. They had negative skintests and a fall $\leq$10% in FEV_1 after challenge with 8 mg/ml methacholine.

Fiberoptic bronchoscopy procedure

Before the bronchoscopy procedure blood samples were taken. After obtaining IVC and FEV_1 prior to the bronchoscopy procedure, asthmatic subjects inhaled 200 μg salbutamol via a spacehaler. After atropine 0.5 mg intramuscularly and local anaesthesia with lidocaine 2% applied to the upper respiratory tract, fiberoptic bronchoscopy was performed with an Olympus BF-IT10 at 16:00 hours and 04:00 hours or vice versa, separated by one week. The guidelines of the ATS were used (9).

Bronchoalveolar lavage

BAL was carried out with the bronchoscope wedged into the lateral segment of the right middle lobe by injecting 10 aliquots of 20 ml sterile phosphate buffered saline (PBS) prewarmed to 37°C and recovered by gentle suction (-40 cm H_2O). The BAL fluid was collected in polypropylene tubes and immediately placed in ice. The BAL fluid was pooled into two fractions: pool 1 with the first 2 x 20 ml instilled PBS, and pool 2 with the remaining 160 ml. The procedures of cell isolation, counting of total and differential cell numbers have been described previously (10).

Analysis of cells from blood and BAL fluid

Subsets and activation state of lymphocytes in blood and BAL fluid from pool 2 were analysed by flow cytometry using a double staining procedure. The cells were incubated with appropriate dilutions of mouse monoclonal antibodies (moabs) conjugated with a fluorochrome (fluorescein isothiocyanate [FITC] or phyco-erythrin [PE]) against CD3 (Leu 4), CD4 (Leu 3a) and CD8 (Leu 2a). CD25 (Tac, α-chain IL-2 receptor) was used in combination with CD4 as an index of CD4+ T-lymphocyte activation. The total leukocyte marker CD45 (HLe-1) and monocyte marker CD14 (Leu-M3) were used to verify the purity of the lymphocytes. FITC and PE conjugated mouse immunoglobulins of matched isotypes (IgG_1, IgG_{2a}) were used as negative controls.

EDTA blood (100 μl) was fixed with 0.05% formaldehyde for 10 minutes at 22 °C centrifugated at 900 g for 2 minutes, then incubated for 15 minutes at 22 °C with the appropriate dilutions of moabs supplemented with 10% AB serum. After lysis of red blood cells with lysing solution (155 mmol/L NH_4Cl, 10 mmol/L KCl, NaAz 0.02%) the cells were washed, resuspended with PBS/BSA 0.5% and allowed to regenerate during 30 minutes at 22 °C.

BAL cell suspension (250 μl) was centrifuged at 500 g at 10 °C for 5 minutes and subsequently incubated at 4 °C for 30 minutes with the appropriate dilutions of moabs with AB serum 5%. After incubation, the cells were washed twice with PBS/BSA 0.5%, centrifugated at 1000 g at 10°C for 2 minutes and 150 μl formaldehyde 0.05% was added for cell fixation.

Flow cytometric measurements were performed with a FACS 440 (Becton Dickinson). In blood, a minimum of 20,000 events were collected for each sample. In BAL, a gate was set for the lymphocytes and at least 10,000 events were counted.

Analysis was performed using a software package (Lysys, Becton Dickinson).

Activated eosinophils in pool 1 of the BAL fluid were identified by immunostaining of acetone-fixed cytocentrifuge preparations with the monoclonal antibody EG2 (Sanbio, Uden, The Netherlands) (11, 12). The slides were counterstained with fresh Mayers' haematoxylin. EG2+ cells were counted in 500 cells at two slides, the average being taken as the definitive number. Only intact and unclustered cells were counted. Total EG2+ cell counts were calculated from the total leukocyte number per ml BAL fluid. EG2+ cells could not be detected in peripheral blood. Blood eosinophil numbers were counted in a Bürker chamber after staining with eosine.

Bronchial biopsy processing and immunostaining

Bronchial biopsies were obtained from subcarinas of alternately the right lower lobe or left lower lobe using a standard fenestrated cup forceps (Olympus, FB-19C). They were immediately placed in isotonic saline, subsequently embedded in TissueTek® and snap-frozen by immersion in precooled isopentane and stored at -80 °C until use.

Immunostaining was performed for activated eosinophils (EG2), T-lymphocytes (CD3) and T-cell subsets (CD4, CD8) and activation (CD25). An immunoperoxidase method was used (13). In short, 4 μm sections were cut, dried and fixed in acetone for 10 minutes at room temperature. After fixation and after each following step, the slides were washed in phosphate-buffered saline (PBS), pH 7.4 for 5 minutes. The slides were first incubated with 50 μl of the monoclonal antibody for 1 hour, then with biotinylated rabbit anti-mouse $F(ab')_2$ fragments 1:300, supplemented with human AB serum 1% for 30 minutes. This was followed by incubation with peroxidase-conjugated streptavidin 1:300, supplemented with human AB serum 1% also for 30 minutes. 3-Amino-9-ethylcarbazole (AEC) together with H_2O_2 was used as reagent for demonstration of peroxidase reactivity. Sections were counterstained with fresh Mayers' haematoxylin.

Quantitation of cells in the bronchial submucosa

Sections were examined in a blinded fashion using a light microscope at a 200 × magnification. They were considered to be representative if the basement membrane had a continuous length of at least 200 μm and the submucosa was at least 100 μm in depth. Cells are present both in a small layer beneath the basement membrane and in the deeper area of the submucosa. At some places cells are grouped in large numbers;

Table 1. Peripheral blood data.[1] Medians with ranges.

	Controls		Asthmatics	
	16:00 h	04:00 h	16:00 h	04:00 h
CD3+	72.0 (56-79)	79.5 (64-88)[2]	80.0 (68-84)	79.5 (72-84)
CD4+	44.5 (31-50)	47.5 (40-58)[2]	46.0 (43-50)	53.5 (36-56)
CD25+/CD4+	6.0 (2-17)	3.5 (0- 7)[2]	6.0 (2-11)	3.5 (1-11)
CD8+	27.5 (25-43)	27.5 (24-33)	26.5 (23-37)	25.5 (22-34)
EOS	88 (22-297)	198 (22-352)	275 (88-462)[3]	314 (231-1002)[3]

1) T-lymphocyte subsets expressed as percentage of total blood leukocytes, eosinophils expressed as total number ($\times 10^6$/L);
2) $P \leq 0.05$ within the group;
3) $P \leq 0.05$ between groups at the same point of time.

elsewhere they are scattered all over the submucosa. We used a semi-quantitative measurement for cell counting underneath the basement membrane and throughout the available submucosa. The scale ranged from no stained cells (score 0) to many stained cells (score 3). At least four sections per subject stained with the same moAb were analyzed in this manner.

Data analysis

Values are presented as medians with ranges. The Mann-Whitney U test was used to compare data between groups, and Wilcoxon's matched signed rank test was applied for within-group analysis. Statistical analysis was performed with SPSS/PC+ V 4.01 statistical package (SPSS Inc., Chicago, IL). P values ≤ 0.05 were considered statistically significant.

Results

Numbers of (activated) eosinophils were always significantly higher in asthmatic subjects than in healthy controls at both points of time in peripheral blood, bronchial submucosa and BAL fluid. The two patients with increased nocturnal airways obstruction did not differ in their inflammatory cell composition from the other asthmatic subjects.

Peripheral blood (table 1)

In healthy subjects the %CD4+ cells increased from 44.5 (31-50) at 16:00 hours to

Table 2. Bronchial biopsy data.[1]

	Controls		Asthmatics	
	16:00 h	04:00 h	16:00 h	04:00 h
CD3+	1 (0-2)	1 (0-1)[2]	1 (0-2)[3]	1 (1-3)[3]
CD4+	1 (1-2)	1 (0-1)	1 (1-2)[3]	2 (1-3)[2,3]
CD25+	0 (0-3)	0 (0-2)	1 (0-2)[3]	1 (0-3)[3]
CD8+	1 (0-2)	1 (1-1)	1 (1-3)[3]	2 (1-3)[3]
EG2+	0 (0-1)	1 (0-2)	1 (0-3)[3]	2 (0-2)[3]

1) Semi-quantitative score of cell types in the submucosa;
2) $P \leq 0.05$ within the group;
3) $P \leq 0.05$ between groups at the same point of time.

47.5 (40-58) ($P \leq 0.05$) at 04:00 hours. At the same time points the %CD25+/CD4+ cells decreased from 6.0 (2-17) to 3.5 (0-7) ($P \leq 0.05$). In asthmatic subjects there were no significant changes in %CD4+ cells and in %CD25+/CD4+ cells. The median number of eosinophils tended to increase at night ($p = 0.07$).

Bronchial submucosa (table 2)

Significantly higher scores in T cell subsets and EG2+ cells were observed in asthmatics as compared to control subjects at both time points. In asthmatic subjects the median score of CD4+ cells was significantly increased at night ($P \leq 0.05$), whereas the same trend was seen in the score of CD8+ cells ($P = 0.06$). The EG2+ cell score was not significantly increased at night.

BAL fluid (table 3)

In asthmatic subjects, the number of EG2+ cells increased significantly at night ($P = 0.03$), whereas the %CD4+ and %CD8+ cells showed opposite trends ($p = 0.07$). These changes were not observed in the control group.

Cell 'traffic' from peripheral blood via bronchial submucosa to BAL fluid

A higher %CD4+ cells in peripheral blood of asthmatics at 04:00 hours was associated with a higher score of CD4+ cells in the bronchial submucosa. In contrast, a

Table 3. BAL fluid data.[1] Medians with ranges.

	Controls		Asthmatics	
	16:00 h	04:00 h	16:00 h	04:00 h
CD3+	88.5 (72-91)	86 (71-95)	86.0 (84-88)	89.0 (82- 92)
CD4+	57.0 (49-64)	57.0 (43-64)	44.0 (35-51)[3]	53.0 (42-58)
CD25+/CD4+	6.0 (4-13)	9.0 (4-20)	7.0 (2-15)	11.0 (4-21)
CD8+	28.0 (17-35)	26.5 (18-29)	39 (33-51)[3]	30.0 (25-44)
EG2+	0.45 (0-1.2)	1.20 (0-13)	3.74 (1-33)[3]	19.1 (9.4-83)[2,3]

1) T-lymphocyte subsets expressed as percentage of total BAL leukocytes, eosinophils expressed as total number ($x10^4$/ml);
2) $P \leq 0.05$ within the group;
3) $P \leq 0.05$ between groups at the same point of time.

higher score of CD4+ cells in the bronchial submucosa was associated with a lower %CD4+ cells in BAL fluid (figure 1). These relationships were not observed in both groups at 16:00 hours. In asthmatics, a positive relationship was observed between %CD25+/CD4+ lymphocytes in peripheral blood and in BAL fluid, and EG2+ cells in bronchial submucosa at 04:00 hours. This relationship was less clear at 16:00 hours. The number of eosinophilic granulocytes in peripheral blood were positively associated with the number of EG2+ cells in the bronchial submucosa in asthmatics at both points of time (figure 2). The same positive relationship existed between EG2+ cells in bronchial submucosa and %EG2+ cells in BAL fluid.

In healthy subjects a higher number of eosinophils in peripheral blood was associated with a higher number of EG2+ cells in bronchial submucosa at 16:00 hours (fig. 4). No other relationships could be demonstrated in healthy subjects or asthmatics.

Discussion

This study compares inflammatory cell changes in peripheral blood, bronchial submucosa and BAL fluid in atopic asthmatic and healthy control subjects at 16:00 hours and 04:00 hours. In this way we have tried to establish an indication for the route of cellular traffic at day and night. Although we realize that the number of subjects in this study is small we were able to find a slight but significant increase of CD4+ cells in the bronchial submucosa of atopic asthmatics at 04:00 hours compared

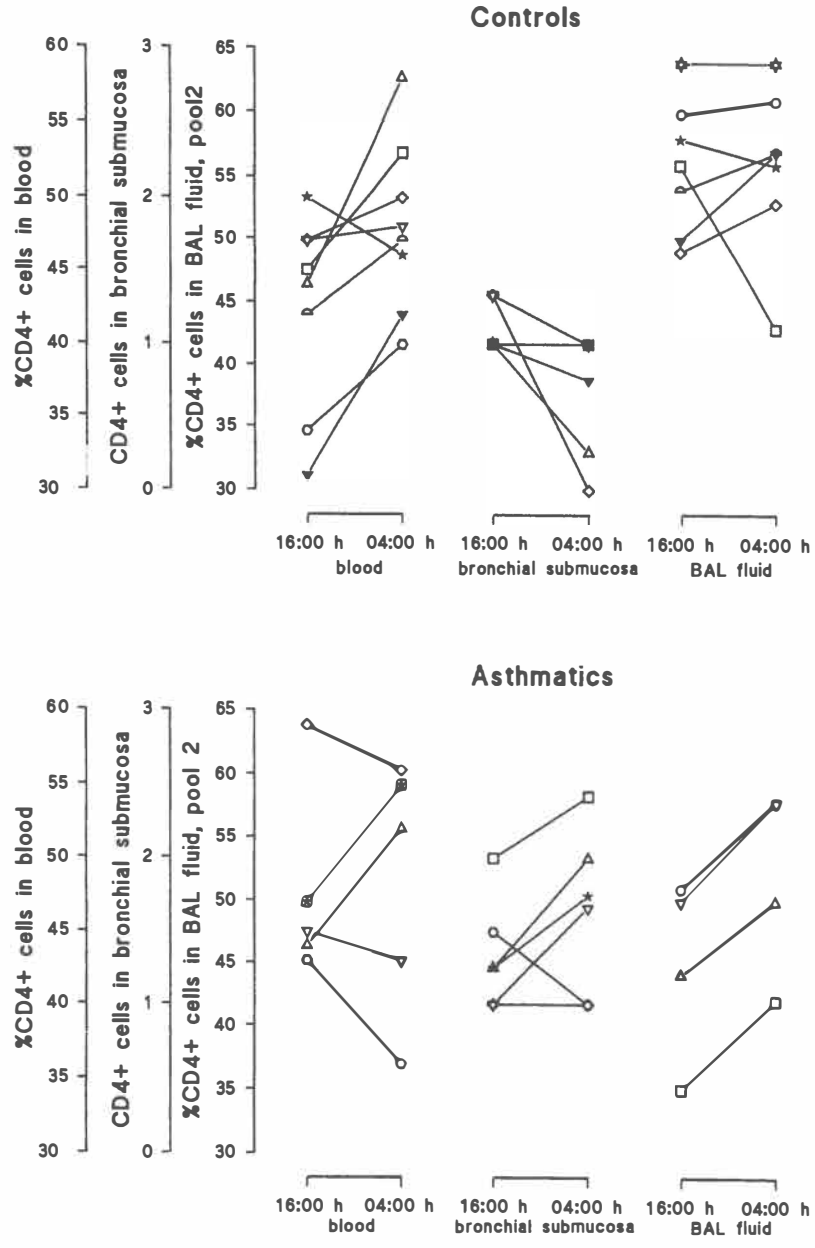

Figure 1. CD4+ cells in peripheral blood, bronchial submucosa and BAL fluid of healthy and asthmatic subjects at 04:00 and 16:00 hours. Each individual has the same marking (e.g. asterisk, triangle) in all figures.

to 16:00 hours. Other day-night differences in T-lymphocyte subsets and eosinophils in bronchial biopsies and BAL fluid of normal subjects and asthmatics have not been found.

Besides the presence of cells in a small layer beneath the basement membrane, inflammatory cells, mainly lymphocytes, were also found in the deeper area of the submucosa. These cells are partly grouped in large numbers, as well as diffusely scattered in the submucosa. Individual cell counting seems not reliable in these cell clusters, but they likely have biological significance. We therefore have included them in our cell counting.

Asthmatics had more T-lymphocytes in their submucosa at 16:00 hours than healthy controls. The difference was even greater at 04:00 hours as healthy controls did not show an increase from 16:00 to 04:00 hours. Numbers of submucosal CD4+ cells are higher in asthmatics than in controls at both points of time (figure 1) and a higher %CD4+ cells in peripheral blood was associated with more CD4+ cells in submucosal biopsies at 04:00 hours. This finding was, however, not observed in healthy individuals (figure 1). Furthermore, a higher number of submucosal CD4+ cells was associated with a lower % of BAL CD4+ cells in asthmatics. These results suggest that, in asthmatics, CD4+ cells are attracted at night from the peripheral blood to the submucosa where they home locally. Tentatively this may be due to local release of cytokines from mast cells, epithelial cells and T-lymphocytes themselves, that keep CD4+ cells at the site of inflammation by adhesion, mediated by β1 integrins to extracellular matrix proteins (14-16).

Asthmatics have higher numbers of eosinophils in peripheral blood than healthy individuals in association with both higher numbers of EG2+ cells in the bronchial submucosa and in BAL fluid. This phenomenon is seen at 16:00 hours as well as at 04:00 hours (figure 2). The finding that a positive relationship exists between % CD4+/CD25+ cells and the number of EG2+ cells in the mucosal biopsies contributes to the hypothesis that eosinophils move from the peripheral blood to the bronchial submucosa, where they become activated by e.g. cytokines from CD25+ lymphocytes (17). In addition, these cytokines may activate endothelium of local vessels with upregulation of VCAM-1 and ICAM-1 enabling chemotaxis of eosinophils and lymphocytes. This process can be facilitated by damaging of the bronchial epithelial layer by degranulation products of the activated eosinophilic granulocyte (18, 19) and due to shedding of the epithelium (1, 20, 21) resulting in an unhampered moving of the

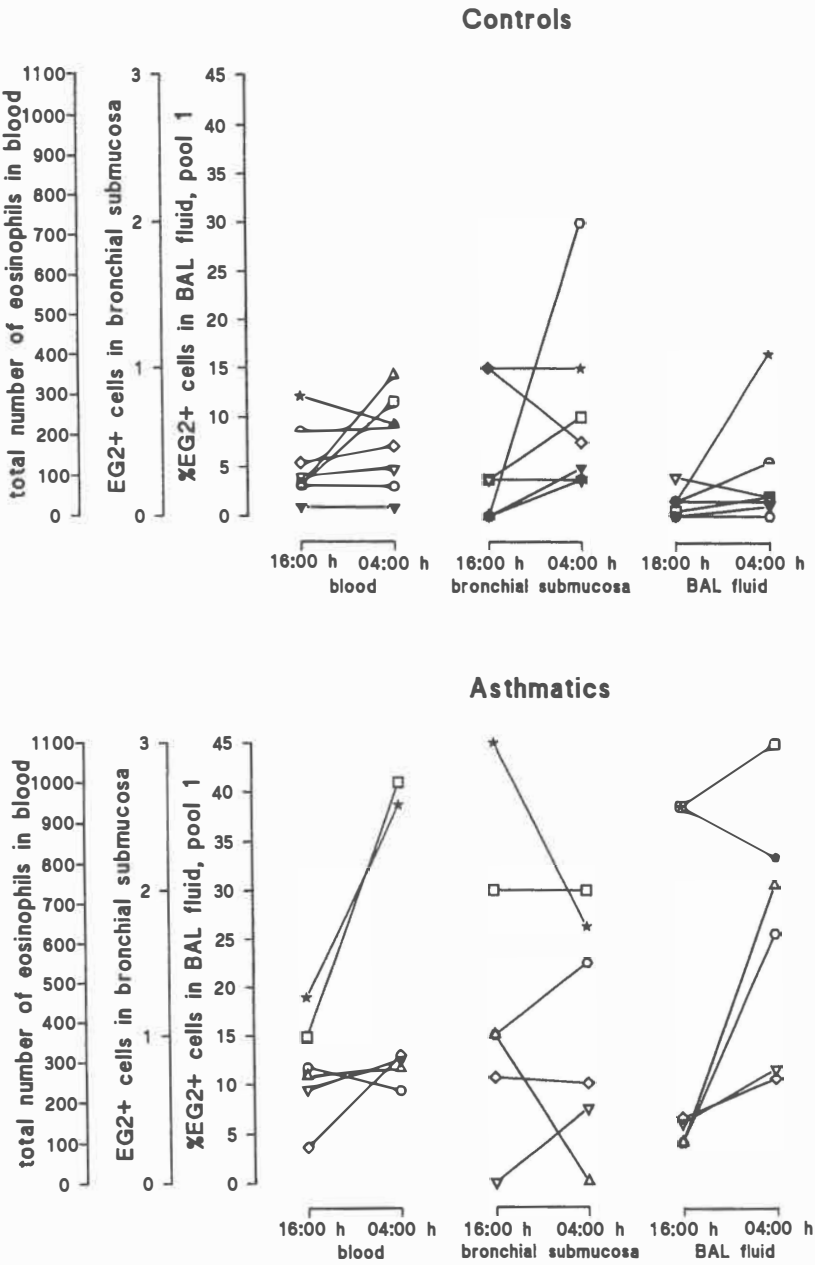

Figure 2. (Activated) eosinophils in peripheral blood, bronchial submucosa and BAL fluid of healthy and asthmatic subjects at 04:00 and 16:00 hours. Each individual has the same marking (e.g. asterisk, triangle) in all figures.

eosinophil through the basal membrane into the airway lumen.

Mackay and colleagues did not find cellular changes in the bronchial submucosa of patients with nocturnal asthma (22). The continued use of inhaled corticosteroids by their patients with nocturnal asthma and the difference in atopic status may offer an explanation for the discrepancy with our observations. As in our study they observed a slight increase in eosinophils in bronchial biopsies of normal subjects at 04:00 hours.

Nocturnal dyspnea may be the consequence of exposure to allergens, like house dust mite, leading to a late asthmatic reaction (LAR) at night (6). Recent studies in atopic asthmatics have reported a negative correlation between the number of CD8+ cells in BAL or bronchial submucosa with the severity of the LAR, suggesting that influx of CD8+ cells may prevent the development of the LAR (2, 23, 24), which is associated with an influx of (activated) eosinophils. Thus, one might expect a negative correlation between CD8+ cells and (activated) eosinophils. We did not find such a relationship between CD8+ cells in the three compartments and EG2+ cells in the bronchial submucosa. A reason for the lack of correlation in our study may be that endogenous factors in nocturnal airflow limitation are more important than atopy and allergen exposure, or that nocturnal house dust mite exposure was less than in case of a laboratory challenge as has been used in the study of Aalbers et al. (24).

In conclusion, inflammatory cell numbers and their activation state in the bronchial mucosa were more pronounced in asthmatics at both points of time. The results of this study together suggest that CD4+ cells are attracted from peripheral blood to the bronchial submucosa where they stay localized at 04:00 hours, whereas eosinophil recruitment from the peripheral blood to the bronchial submucosa occurs at both time points, where they further move on into the airway lumen. More studies are required to determine the exact role of inflammation in asthmatics with a nocturnal increase in airways obstruction.

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Chapter 8

INFLAMMATION IN NOCTURNAL ASTHMA?

D.S. Postma, Y. Oosterhoff, W.M.C. van Aalderen, H.F. Kauffman,
J.B. Wempe, G.H. Koëter

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Introduction

It has been recognised since centuries that patients with asthma may suffer from severe attacks of asthma at night (1). This is largely the consequence of an increased airways obstruction at night, reflecting bigger swings in airway diameter than observed in many other asthmatic patients and in healthy individuals. As such, the term 'nocturnal asthma' suggests that it may be a special form of asthma, only occurring at night. But it is still debatable whether nocturnal asthma is a unique entity in asthma or merely a representation of more overall severe illness. In this article we will survey the present knowledge on this topic and try to show what evidence exists to either call nocturnal asthma a different entity or just a different expression of one and the same disease. Attention will especially be focused on the role of inflammation in this respect.

Trigger factors

Several factors trigger bronchoconstriction at night (2,3). Sleep seems to synchronise the circadian rhythm: in shift- workers peak expiratory flow (PEF) varies with the time of sleeping rather than the time of day. However, recent studies suggest that there is more to sleep than at first sight (4,5). Many other factors have been incriminated in the occurrence of airways obstruction at night. One of them is the increase in airways responsiveness to methacholine and histamine at night (6,7), leading to recurrent nocturnal dyspnea, life-threatening nocturnal attacks, or even death (8). Both endogenous factors, related to their chronobiological rhythms, such as the autonomic nervous system activity, circulating adrenaline, cortisol and release of cellular mediators, and environmental factors, such as allergen and cigarette smoke exposure are supposed to contribute to the increase in airways obstruction (9) and airways responsiveness at night (figure 1). The mechanisms by which these factors operate are not fully understood, but recent reports suggest that inflammatory processes may be the key factor to it (2,3).

Although many cells can contribute to an inflammatory process, mast cells and eosinophils are considered as principal effector cells in allergic asthma (10). Upon activation, mast cells in the lung can release histamine and prostaglandin D_2 (PGD_2), both being potent constrictors of the airways. Furthermore, prostanoids play a modulatory role in airways responsiveness (11). Another prime cell thought to be important in asthma is the eosinophil. Upon allergen challenge it has been observed that the number of eosinophils increases in bronchoalveolar lavage (BAL) fluid during the

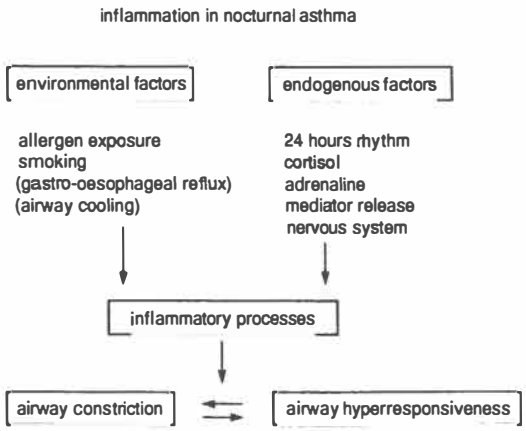

Figure 1. Schematic drawing of trigger factors of nocturnal asthma.

late bronchoconstrictive reaction, the particular moment of increased airways hyperresponsiveness (12). It is now generally acknowledged that the release of preformed mediators, such as eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN), as well as other mediators may result in airway damage, oedema, and airways hyperresponsiveness. The first reports suggesting a role for the eosinophils in nocturnal worsening of airways hyperresponsiveness showed the presence of higher numbers of eosinophils in the peripheral blood at night (13). A higher number of circulating eosinophils was associated with a lower mean circadian PEF level. Furthermore, a larger circadian variation in eosinophil numbers was related to bigger swings in PEF over day and night. But the mere presence of higher numbers in peripheral blood eosinophils may not simply explain a higher severity of airways responsiveness at night.

What then is the evidence to relate inflammation, and in particular above mentioned cells to the nocturnal increase in airways hyperresponsiveness? ***Indirect evidence*** may stem from studies on circadian variation of airways responsiveness to 'direct' and 'indirect' stimuli (14), numbers and activation of peripheral blood inflammatory cells (15-19) and modulation of the former and latter by different treatment regimens (17), known to affect airways hyperresponsiveness and inflammation in different ways. More ***direct evidence*** may be gained by studies on number and activation of cells obtained by bronchoalveolar lavage (20-23). Finally, above data combined with airways biopsies have to show the difference between

daytime and nighttime traffic and activation of inflammatory cells from peripheral blood to the site of their action in airway wall tissue.

Indirect evidence for inflammation in nocturnal asthma.

It is generally felt that airways hyperresponsiveness in asthma may reflect an increase in airways inflammation. There are now a few studies showing that airways responsiveness to histamine and methacholine are increased at night (6,7,14). This appears not only to happen when asthmatic patients experience a fall in FEV_1 at night, but also in asthmatics who have a stable circadian rhythm in airway diameter (7). This suggests that the change in airway diameter is not the sole primum movens of this increased airways responsiveness to histamine at night. Airways responsiveness is nowadays measured with different stimuli. Methacholine is thought to cause airways obstruction mainly by a direct effect on receptors of airway smooth muscles, whereas stimuli like adenosine monophosphate (AMP) and propranolol are thought to primarily act on other cells, thus initiating processes that indirectly lead to smooth muscle contraction. Recent studies suggest that AMP acts as an airway constrictor by histamine release from mast cells and by local peptidergic reflexes (23). It is therefore interesting to observe that AMP responsiveness is far more affected at night than methacholine (and propranolol) responsiveness in patients with nocturnal increase in airways obstruction (figure 2) than in those who have a stable circadian airway diameter (14). Moreover, a greater circadian change in AMP responsiveness was related to a larger circadian PEF variation (14). Another important observation in this study is, that individuals without nocturnal asthma also have less daytime responsiveness to AMP and propranolol, both indirect stimuli of airway smooth muscles (figure 2). This higher susceptibility to indirect stimuli in patients with nocturnal asthma suggests that mast cell activation rather than changes in smooth muscle contractility play a role in this respect. One has to realise, however, that AMP hyperresponsiveness is not solely more prominent at night, but also at daytime in the patient group with nocturnal asthma.

This observation is an important one, as inhaled corticosteroids are threefold more protective against an AMP-provocation than against a methacholine provocation in asthmatic individuals (24). It has been shown that inhaled corticosteroids, well known for their anti-inflammatory properties, are also very effective in reducing nocturnal asthma symptoms (25). Moreover, the circadian variation in airways hyper-

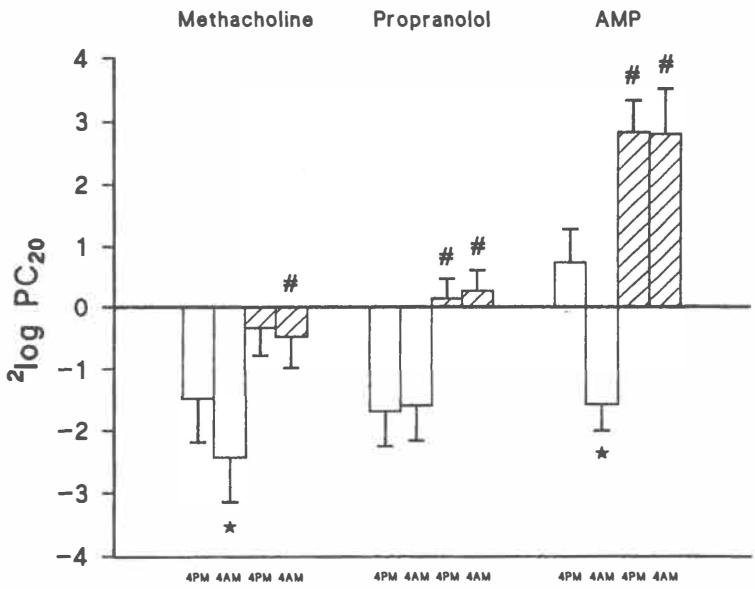

Figure 2. Airways responsiveness to AMP, methacholine and propranolol at 4 PM and 4 AM in patients with (open bars, NA^+) and without (hatched bars, NA^-) nocturnal asthma. * $p < 0.05$ 4 PM vs. 4 AM; # $p < 0.05$ NA^+ vs. NA^-.

responsiveness was more reduced with inhaled corticosteroids than with a long acting oral beta-agonist (25). The increased sensitivity to indirect stimuli at night and the beneficial effect of corticosteroids on nocturnal symptoms may support an important role for inflammation in nocturnal asthma. One has to realise again that inhaled corticosteroids do not completely suppress all variation in responsiveness to histamine over 24 hours. Nevertheless, both the nocturnal increase in numbers of eosinophils and their activation parameters ECP and EDN were reduced at night by twice daily inhalation of corticosteroids (figure 3), suggesting at least a reduction in eosinophil activation (17).

Contradictory results have been reported on variations of blood eosinophil numbers and their activity between 4 AM and 4 PM in nocturnal asthmatics. Fitzpatrick and coworkers (18) reported decreased eosinophil numbers, along with reduced serum ECP levels during the night and suggested these findings to be compatible with the involvement of a late asthmatic reaction in the pathogenesis of nocturnal asthma. It cannot be excluded, however, that the use of inhaled and oral corticosteroids in the latter study may have affected the results (17). Nevertheless, since in Fitzpatrick's

study the nocturnal asthmatics had large nocturnal falls in PEF and lower baseline functions as compared to other studies, it may be concluded that the severity of the asthmatic disease may explain differences in findings of circadian changes in blood eosinophil numbers and activation products reported in the various studies. Calhoun et al. (16) have also found some indications for eosinophil activation in peripheral blood of patients with nocturnal asthma. These patients had larger numbers of peripheral blood eosinophils both at 4 AM and 4 PM than asthmatics without a nocturnal fall in FEV_1 of more than 15%, suggesting more severe asthma in the former group. However, they did not find a difference between patients with and without nocturnal asthma with respect to their severity of asthma in daytime, as measured by FEV_1 and responsiveness to histamine at 4 PM. They therefore suggested that circulating cortisol and adrenaline was sufficient enough to suppress the ongoing diurnal eosinophil activation even in the group with the highest number of eosinophils. At night this was not the case, as they observed a significant increase of low-density eosinophils at 4 AM only in the 5 individuals with nocturnal asthma. Another feature of eosinophils with enhanced inflammatory potential is a greater *in vitro* survival (26). Calhoun and coworkers showed that eosinophils from asthmatics generally had greater survival half-life than those from normal subjects (16). The difference between 4 AM and 4 PM survival did, however, not reach significance in patients with nocturnal asthma, possibly due to the low number of subjects involved.

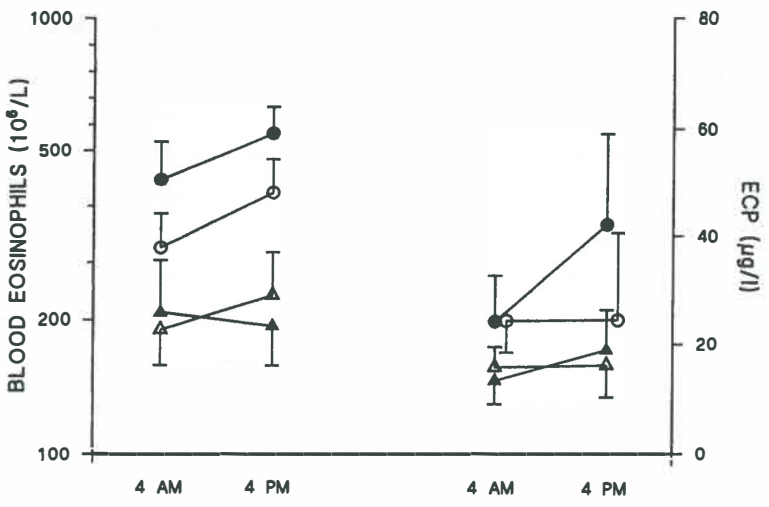

Figure 3. Effect of inhalation corticosteroids (triangle) versus placebo (circle) on peripheral eosinophil counts and ECP levels in patients with (closed) and without (open) nocturnal asthma.

Direct evidence for inflammation in nocturnal asthma.

Conflicting results with regard to the role of inflammation in nocturnal asthma has been gained by BAL. Martin et al. (20) were the first to present their results in patients with and without nocturnal asthma. They demonstrated significant increases in numbers of BAL-eosinophils and -lymphocytes, in association with total white cell counts and neutrophil numbers. In contrast, Jarjour and colleagues (21) did not find changes in BAL cell numbers in their subjects with nocturnal asthma, which corresponds with our own recent findings (22). Comparing patient characteristics of these two studies and ours, the nocturnal asthmatics in Martin's study generally have more severe asthma, with lower mean FEV_1 values and higher circadian PEF-variations at home. It is therefore possible that only in severe nocturnal airways narrowing an association can be found with pronounced infiltration of inflammatory cells into the airways, as recovered by BAL fluid. Furthermore, in both other studies most of the subjects used theophyllines at the time of investigation, whereas our patients did not use maintenance therapy from four weeks before the start of the study. Although Martin reported that the mean serum theophylline concentrations at 4 PM and 4 AM did not differ, it is not clear whether individual differences in serum concentrations were observed. Changes in theophylline levels are known to modulate blood eosinophil numbers (27) and it is not excluded that this may have affected airway leukocyte numbers in their study as well. Finally, it is quite clear from the individual data presented by Martin and coworkers (20), that many patients with a severe nocturnal decrease in FEV_1 did not show an increase in eosinophil or neutrophil numbers at night.

In our own study, no specific variations in inflammatory cells occurred in asthmatics with an increased circadian PEF-variation and most eosinophil and mast cell activation parameters between 4 AM and 4 PM were found unable to differentiate them from asthmatics without nocturnal asthma. For instance the histamine levels in the BAL fluid in both asthma groups of our study were increased above the levels observed in normals, but we could not differentiate between nocturnal asthma and non-nocturnal asthma. This has not been found in an older study (28), where serum histamine was increased at night in patients with nocturnal asthma. However, the technique to measure serum histamine in that study may have resulted in artificial data. Nevertheless, urinary histamine was also increased in children with nocturnal asthma (29). In contrast with the histamine data, we found increased PGD_2 levels specifically in the asthmatics with increased circadian PEF-variation, higher levels being present both

day and night. An important finding is, that PGD_2 levels did not significantly increase at night in patients with nocturnal asthma. PGD_2 has been detected in BAL fluid of stable atopic asthmatics (30), subsequently increasing during immediate airways obstruction and 19 hours after allergen challenge (31,32). Prostanoids are airway constrictors equipotent to histamine and have been implicated in the increase in airways responsiveness, as inhalation of a non-contractile dose of PGD_2 enhanced airways responses to subsequently inhaled histamine and methacholine (33). The increased levels of prostanoids in our group with nocturnal asthma may thus contribute to the increased circadian fluctuation in airways responsiveness.

Today no data have been published on histology in nocturnal asthma. Thus, differences in outcome of results with BAL and biopsies, as shown in the late bronchial obstructive reaction after allergen challenge (34), are also possible in the case of nocturnal asthma. Results of such ongoing studies have to be awaited to finally support the now available data, that inflammatory processes play a role in nocturnal asthma, and that this reflects more severe asthma. Furthermore cytokine profiles have as yet not been presented in BAL fluid or biopsies.

In summary, at present there is some indirect evidence for increased **nocturnal** inflammation in patients suffering from nocturnal asthma:

1) circulating eosinophil numbers and activation, as reflected by increased levels of ECP and EDN and low-density eosinophils are increased at night;
2) circulating histamine levels are increased at night;
3) hyperresponsiveness to AMP at night is increased compared with methacholine;

However, most results of various studies point into the direction of nocturnal asthma being an expression of more severe asthma:

1) AMP and propranolol responsiveness, both indirect measures of airways hyperresponsiveness is lower both at 4 AM and 4 PM in asthmatics with nocturnal asthma than those without nocturnal asthma;
2) patients with nocturnal asthma have higher circulating numbers of eosinophils both at 4 AM and 4 PM than those without nocturnal asthma, and eosinophil survival is not different at these time points;
3) patients with nocturnal asthma have higher PGD_2 levels in BAL both at 4 AM and 4 PM than those without nocturnal asthma, but no significant difference between these two time points;

4) two studies have shown no difference in BAL-eosinophil numbers and activation parameters at night in nocturnal asthma;
5) histamine levels in BAL-fluid are comparable in daytime and at night in patients with and without nocturnal asthma;
6) inflammatory mediators in BAL are higher than in normals, but not different between patients with and without nocturnal asthma.

Thus, patients with nocturnal asthmatic symptoms show an overall increased burden of mediators released from mast cells and other inflammatory cells.

In conclusion, we feel that the term 'nocturnal asthma' is misleading, in that it is not a unique entity in certain patients with asthma. We prefer, in view of above mentioned arguments, to consider 'nocturnal asthma' as a mere expression of more severe asthma. Thus we suggest the term nocturnal asthma to be changed in ' asthma with nocturnal symptoms'.

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Chapter 9

AIRWAYS RESPONSIVENESS TO ADENOSINE 5'- MONOPHOSPHATE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE IS DETERMINED BY SMOKING

Ytske Oosterhoff, Jan W. de Jong, Maarten A.M. Jansen,
Gerard H. Koëter, Dirkje S. Postma

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Abstract

In contrast to methacholine, a stimulus which induces airways constriction mainly by 'direct' stimulation of airways smooth muscle cells, AMP airways responsiveness reflects 'indirectly' induced airways narrowing via inflammatory or neural reflex mechanisms. In order to determine inflammatory contribution to airways narrowing in COPD, we performed AMP and methacholine inhalation provocation tests in nonatopic subjects with COPD and compared the results with those obtained from atopic nonsmoking asthmatics and from healthy smoking volunteers.

AMP caused airways narrowing in all but two subjects with COPD and in only three of the 12 healthy smoking subjects. Patients with COPD were significantly more responsive to AMP and methacholine than healthy smoking volunteers. Geometric mean PC_{20} AMP was significantly lower in the smokers with COPD (7.2 mg/ml) than in the nonsmokers with COPD (58.5 mg/ml), whereas PC_{20} methacholine values and baseline FEV_1 were comparable. In the nonatopic nonsmoking subjects with COPD, PC_{20} AMP was significantly higher than in the atopic nonsmoking asthmatics (3.8 mg/ml), whereas they responded similar to methacholine provocation.

These results indicate that most subjects with COPD respond to AMP provocation and that smoking determines the degree of airways responsiveness to AMP in COPD. We suggest that increased susceptibility to mediator release by mast cells or neural reflex mechanisms are involved in AMP-induced airways constriction in asthma and in COPD.

Introduction

Airways hyperresponsiveness (AH) is defined as an exaggerated airways constrictive reaction on exposure to a provoking stimulus and is a characteristic feature in subjects with chronic obstructive pulmonary disease (COPD) and asthma (1, 2). However, it is recognised that the kind of stimulus used matters and that differences in responsiveness between COPD and asthma exist when 'direct' and 'indirect' stimuli are used for provocation (3).

Methacholine is regarded as a direct stimulus that mainly acts via muscarinic receptors on smooth muscle cells. Upon methacholine challenge airways constriction is induced in both COPD and asthma, though generally in asthma the sensitivity is higher and the slope of the dose-response curve steeper than in COPD (4, 5).

In contrast, after provocation with 'indirect' stimuli, i.e., stimuli that act

primarily on other cells, evoking processes that secundarily lead to airways smooth muscle contraction, an airways constrictive response can be induced in asthmatics, but generally not in subjects with COPD. For instance, no response was found in COPD after provocation with propranolol, methoxamine, cold air hyperventilation and polymyxin B, stimuli which are thought to act partly via mast cell release (3), nor after provocation with SO_2, a trigger that is supposed to act via nervous mechanisms (6). Results from these studies suggest that 'indirect' airways narrowing induced by inflammatory processes or neural reflex mechanisms in the airways do not play a major role in COPD.

Nevertheless, inflammatory changes have been described in lung tissue obtained from both asthmatics and patients with COPD (7, 8), although differences exist in factors eliciting the inflammatory processes, as well as in the site of airway inflammation and type of infiltrating cells. In asthma, hypersensitivity to allergens is supposed to contribute to the observed changes, which are found throughout the airways. The infiltrating cells consist of activated eosinophils and lymphocytes. In contrast, in COPD cigarette smoke contributes to the inflammatory changes. These changes are mainly located in the smaller airways. An infiltration of mononuclear cells in the airways predominates (9). Besides, smoking induces infiltration of macrophages, neutrophils and mast cells in the airways (10-12). Higher N^{τ}-methylhistamine levels have been found in the urine of subjects with COPD compared with normals (13) and increased tryptase and histamine levels in the bronchoalveolar lavage fluids of healthy smokers (14), suggesting an increased susceptibility of airway mast cells to release their mediators.

In order to determine inflammatory contribution to airways narrowing in COPD, we investigated airways responsiveness to another 'indirect' stimulus, adenosine 5'-monophosphate (AMP). Evidence has been shown for involvement of mast cell release in AMP-induced airways constriction (15-17). AMP is known to induce a concentration-related airways constriction in atopic and nonatopic asthmatic subjects as well as atopic nonasthmatic subjects (18, 17). Results in COPD, however, have as yet scarcely been published (19). Therefore, we performed AMP and methacholine inhalation provocation tests in nonatopic elderly smoking and nonsmoking subjects with a diagnosis of COPD and compared the results with those obtained from atopic young nonsmoking subjects with asthma and healthy nonatopic elderly smoking subjects.

Methods

Subjects

Thirty nonatopic smoking and nonsmoking subjects with a diagnosis of COPD, 16 atopic nonsmoking subjects with asthma, and 16 nonatopic smoking healthy subjects were investigated. They were recruited from our outpatient clinic of the Department of Pulmonary Diseases and from advertisements in local newspapers, respectively. The study was approved by the local hospital ethics committee. All subjects signed an informed consent.

All subjects had no concomitant diseases and no upper respiratory tract infection within 4 wk before the study. None of the subjects with COPD or asthma had received oral corticosteroids within 2 months before the study. Inhaled corticosteroids, cromoglycate and antihistamine medication, if used, were stopped at least 2 wk before onset of the study. Theophylline was discontinued for 48 h. Nonsmoking was defined as not having smoked during the past 3 yr. Further selection criteria at enrollment in the study were as follows.

COPD group: (*1*) age $\geq$ 40 yr; (*2*) a history of chronic airways obstruction consistent with the clinical diagnosis of COPD, as defined according to the criteria of the American Thoracic Society (1); (*3*) FEV_1 < 70% predicted, and postbronchodilator FEV_1 < 80% predicted; (*4*) airways hyperresponsiveness to histamine, defined as the provocative concentration of histamine that caused a 20% fall in FEV_1 (PC_{20}), of $\leq$ 8 mg/ml (2-min inhalation, 5-min interval); (*5*) no atopy: negative history of allergy plus negative intracutaneous tests against 16 common aeroallergens (Diephuis Laboratories, Groningen, The Netherlands) plus negative specific IgE against house dust mite (Phadezym^R RAST; Pharmacia, Uppsala, Sweden).

Asthma group: (*1*) age between 18 and 45 yr; (*2*) a history of episodic dyspnea or wheezing consistent with the clinical diagnosis of asthma (1); (*3*) FEV_1 > 60% predicted, and postbronchodilator FEV_1 $\geq$ 80% predicted; (*4*) airways hyperresponsiveness to histamine $\leq$ 8 mg/ml; (*5*) atopy: positive intracutaneous tests against house dust mite or two other of the tested common aeroallergens, or elevated specific IgE against house dust mite (RAST score $\geq$ 0.7 PRU/ml).

Smoking control group: (*1*) age $\geq$ 40 yr; (*2*) no previous history of respiratory disease; (*3*) FEV_1 $\geq$ 80% predicted; (*4*) airway hyperresponsiveness to methacholine > 8 mg/ml; (*5*) no atopy: negative history of allergy plus negative intracutaneous tests against 16 common aeroallergens; (*6*) no use of medication.

Study design

The subjects attended the laboratory on two visits at the same time of day. At the first day (Day 1) a methacholine provocation test was performed, followed after 2 to 9 days by an AMP provocation test (Day 2). Inhaled bronchodilators, if used, were omitted at least 8 h before each provocation.

Methacholine and AMP inhalation-provocation tests

Airflow was measured using a calibrated water-sealed spirometer according to standardized guidelines (20). First, prechallenge FEV_1 was measured until three reproducible recordings were obtained. The best of three was used for analysis. Reference values are those of the European Community for Coal and Steel (21).

Inhalation provocation tests were performed according to a 2-min tidal breathing method adapted from Cockcroft and coworkers (22). Solutions of methacholine and AMP (Sigma Chemical Co., St. Louis, MO) were administered at room temperature as aerosols generated from a starting volume of 3 ml in a DeVilbiss 646 nebulizer (DeVilbiss Health Care Inc, Somerset, PA), connected to the central chamber of an

Table 1. Subject characteristics.

	Control subjects *(Smokers)*	Patients with COPD *(Smokers)*	Patients with COPD *(Nonsmokers)*	Patients with Asthma *(Nonsmokers)*
Number	12	19	11	16
Sex, M/F	8/4	19/0	10/1	8/8
Age, yr	56.7 ± 3.8	59.7 ± 8.5	62.9 ± 6.7[1]	28.8 ± 7.1
Pack-years	24.9 ± 10.0	24.4 ± 10.7	15.5 ± 15.3	?
Cigarettes/day, n	19.8 ± 10.0	12.8 ± 10.4	-	-
Atopy	-	-	-	+
FEV_1/VC	0.76 ± 0.11	0.44 ± 0.09[2]	0.50 ± 0.12[2]	0.72 ± 0.11
Prechallenge FEV_1, % predicted:				
Day 1	106.4 ± 12.2	48.1 ± 11.7[2]	50.8 ± 10.4[2]	87.6 ± 13.8
Day 2	107.3 ± 10.9	46.4 ± 10.1[2]	50.3 ± 10.8[2]	87.9 ± 17.0

1 $p = 0.04$, compared with control group;
2 $p < 0.001$, compared with control group and asthma group.

inspiratory and expiratory valve box with an expiratory aerosol filter. Solution output was 0.13 ml/min. After inhalation of 0.9% sodium chloride solution, doubling concentrations of 0.03 - 256 mg/ml (0.15 - 1,308 mmol/L) methacholine chloride or 0.04 to 320 mg/ml (0.11 - 913 mmol/L) AMP were inhaled. FEV_1 was measured 30 and 90 s after each inhalation. The challenge was discontinued when FEV_1 had fallen by $\geq$ 20% of the prechallenge level or when the highest concentration had been administered. PC_{20} values were calculated by linear interpolation between the last two data points of the logarithmic concentration response curve. If at the highest concentration administered FEV_1 had fallen < 20% of the prechallenge level, the doubling concentration of the highest concentration administered was used as PC_{20} value, or the PC_{20} value calculated by linear extrapolation of the logarithmic concentration response curve if the FEV_1 had fallen > 15% at the highest concentration administered.

Data analysis

Values are presented as mean (SD). All analyses were performed with the SPSS/PC+ V4.01 software package (SPSS Inc., Chicago, IL). PC_{20} values were analyzed after base-2 logarithmic transformation, a change of 1 unit in $\log_2 PC_{20}$ representing 1 doubling dilution. Differences in prechallenge FEV_1, PC_{20} values and other parameters between the groups were compared by using the Mann-Whitney U test for unpaired observations. After checking for normal distributions, correlations between FEV_1% predicted and PC_{20} values in the COPD and asthma groups were performed using Pearson's correlation test; p values less than 0.05 were considered statistically significant.

Results

Subjects

Subject characteristics are listed in table 1. The subjects with COPD were divided into smoking and nonsmoking groups. Between the COPD groups no significant difference was found in age, pack-years ($p=0.09$), mean baseline FEV_1% predicted and mean FEV_1/VC values.

Age and cumulative index of pack-years were not significantly different between the smoking subjects with COPD and the smoking control subjects, whereas mean daily number of cigarettes used in the smoking COPD group tended to be lower than in the

healthy control group ($p = 0.08$). As expected, baseline FEV_1% predicted and FEV_1/VC were significantly lower in both COPD groups as compared to the healthy control and the asthma group ($p < 0.001$).

On the two different study days, mean baseline FEV_1% predicted was not significantly different within the four groups (table 1).

Methacholine provocation

Geometric mean PC_{20} methacholine values did not differ significantly between both COPD groups and the asthma group (figure 1). The geometric mean value in the nonsmoking COPD group was 0.50 mg/ml; in the smoking COPD group it was 0.35 mg/ml, and in the asthma group it was 0.56 mg/ml. The geometric mean PC_{20} methacholine value in the smoking healthy control group was 180 mg/ml ($p < 0.001$). The methacholine provocation was continued up to a concentration of 256 mg/ml, but five of the 12 control subjects did not respond to the highest concentration administered.

AMP provocation

All but two subjects with COPD responded with airways narrowing upon AMP provocation. The two nonresponders were nonsmokers: one ex-smoker and one lifetime nonsmoker. The geometric mean PC_{20} AMP value in the smoking COPD group was significantly lower than in the nonsmoking COPD group (figure 1). The geometric mean values were 7.16 and 58.49 mg/ml, respectively ($p = 0.002$), equalling a difference of 3.0 doubling dilutions (DD).

The geometric mean PC_{20} AMP value in the nonsmoking asthma group (3.76 mg/ml) was also significantly lower as compared to the nonsmoking COPD group ($p < 0.001$), equalling 4.0 DD. Although the geometric mean PC_{20} AMP in the asthma group was lower as compared to the smoking COPD group (0.9 DD), this was not statistically significant.

In the smoking healthy control group nine of the 12 subjects did not respond to the highest AMP concentration administered. The geometric mean PC_{20} AMP value (375 mg/ml) was significantly higher as compared to the smoking and nonsmoking COPD groups ($p < 0.001$), equalling 5.7 and 2.7 DD respectively.

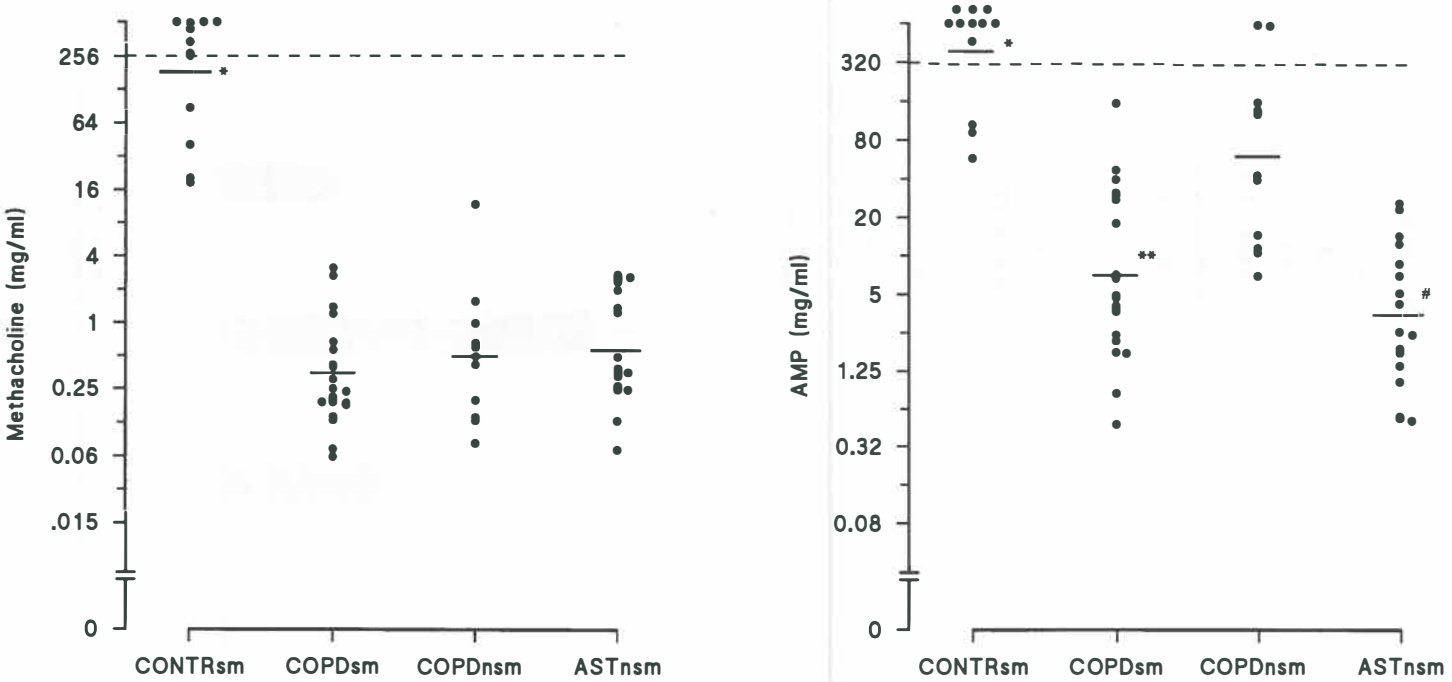


Figure 1. PC_{20} methacholine (*left*) and PC_{20} AMP (*right*) values of smoking control subjects (CONTRsm), smoking subjects with COPD (COPDsm), nonsmoking subjects with COPD (COPDnsm) and nonsmoking asthmatics (ASTnsm). One unit in PC_{20} represents 1 doubling dilution. Individual values are represented by closed circles. Mean values, expressed as geometric mean, are represented by horizontal bars. Dashed line indicate highest concentration administered. Left: * $p < 0.001$ compared with the other three groups. Right: * $p < 0.04$; ** $p = 0.002$; # $p < 0.001$ compared with COPD nsm.

AMP/methacholine ratio

The mean $\log_2$ (PC_{20} AMP/ PC_{20} methacholine) values, calculated on a molar base, differed significantly between all four groups ($p \leq 0.02$) (figure 2). The $\log_2$ value in the asthma group was 1.91, indicating that the asthma group was 3.8 times less responsive to AMP than to methacholine. Calculated from the mean $\log_2$ values in the other groups, the smoking and nonsmoking COPD groups were 11.5 respectively 65 times less responsive to AMP than to methacholine in the smoking COPD group, whereas the smoking healthy control group responded equally to both stimuli (AMP/methacholine ratio: 1.1).

Relationship between airways responsiveness and baseline FEV_1

Regression lines of PC_{20} values expressed on a molar basis with FEV_1% predicted from both COPD groups and the asthma group are shown in figure 3. FEV_1% predicted ranged from 33 to 66% in the nonsmoking COPD group, from 28 to 68% in the smoking COPD group and from 45 to 111% in the asthma group. Therefore, interpretation of the results is limited by the small range of FEV_1% predicted, especially in the COPD groups. In the control group no regression lines were made,

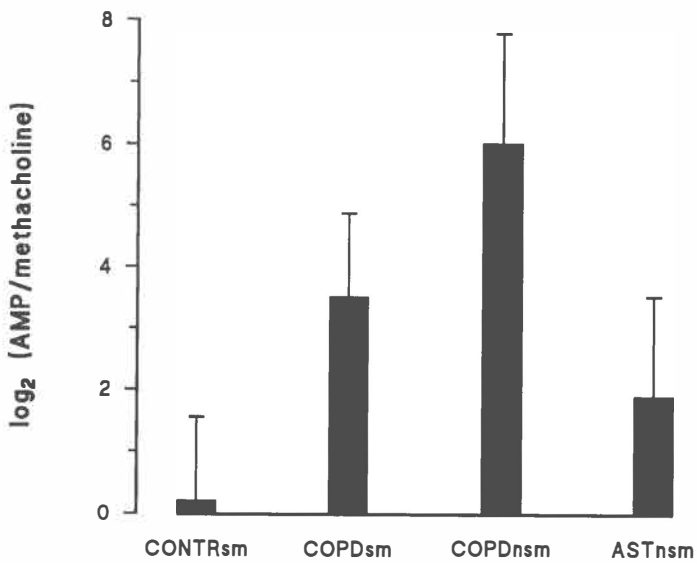


Figure 2. Mean $\log_2$ (PC_{20} AMP/ PC_{20} methacholine) values calculated on molar basis in smoking control subjects (CONTRsm), smoking subjects with COPD (COPDsm), nonsmoking subjects with COPD (COPDnsm) and nonsmoking asthmatics (ASTnsm). Mean values of all groups differed significantly from each other ($p \leq 0.02$).

as many subjects did not respond to the highest administered provocation concentrations of methacholine and AMP.

PC_{20} methacholine and FEV_1% predicted were significantly correlated in the asthma group ($r=0.72$, $p=0.002$), but not in the smoking COPD group ($r=0.42$, $p=0.07$) or in the nonsmoking COPD group ($r=0.12$, NS).

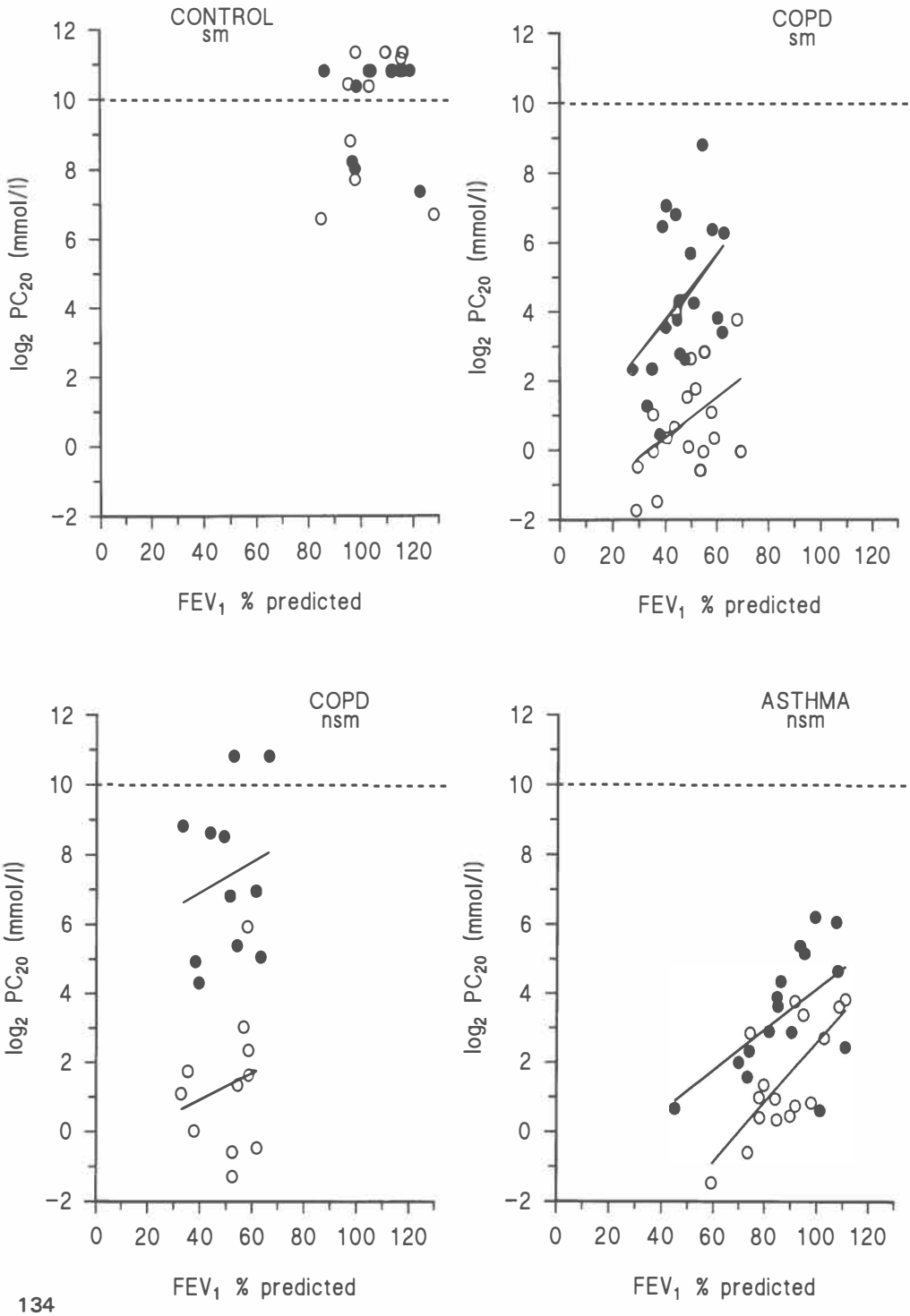
PC_{20} AMP and FEV_1% predicted were also significantly correlated in the asthmatic group ($r=0.56$, $p=0.02$), but not in the smoking COPD group ($r=0.44$, $p=0.06$) or in the nonsmoking COPD group ($r=0.2$, NS).

Discussion

This study demonstrates that AMP provocation caused airways narrowing in all but two of the subjects with COPD. Besides, smoking subjects with COPD were significantly more sensitive to AMP provocation than were nonsmokers with COPD, whereas most smoking healthy volunteers did not respond to AMP provocation.

AMP was much less potent in causing an airways constrictive response in the nonsmoking COPD group than in nonsmoking asthmatics, while the sensitivity to methacholine was similar in these groups. Though generally the sensitivity to methacholine has been reported to be higher in asthmatics than in patients with COPD (4, 5), the similar sensitivity in the asthma and the COPD groups in this study can probably be explained as a consequence of different baseline FEV_1 values between both groups, the values being lower in the COPD group. However, as the FEV_1 values in our COPD group had a narrow range, we cannot deduce with any certainty a conclusion from the nonsignificant correlation between FEV_1 and the PC_{20} AMP or methacholine.

All asthmatic subjects were atopic in our study, whereas the subjects with COPD were nonatopic. One might therefore assume that hypersensitivity to environmental antigens explains the increased sensitivity to AMP in the asthma group. Indeed, Aalbers and coworkers (23) have demonstrated that allergen exposure temporarily increases the AMP airways responsiveness in atopic nonsmoking asthmatics. Moreover, the sensitivity to AMP provocation is increased in both asthmatic and nonasthmatic atopic individuals when compared with nonatopic nonasthmatic subjects, the sensitivity in the asthmatic group being higher than in the nonasthmatic atopic group (18). However, hypersensitivity to environmental antigens is not the sole determinant of AMP responsiveness, as it is has been shown that the sensivity to AMP in nonatopic asthmatics is increased to the same degree as in


CONTROL
sm
COPD
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COPD
nsm
ASTHMA
nsm
log2 PC20 (mmol/l)
FEV1 % predicted
12
10
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6
4
2
0
−2
0 20 40 60 80 100 120

atopic asthmatics (24). This suggests that other (possibly inflammatory) factors also determine the degree of AMP sensitivity in asthma and these might underlie the AMP responsiveness in our COPD patients as well.

A second important finding in this study is that in subjects with COPD the sensitivity to AMP was significantly higher in smokers than in nonsmokers, whereas the groups had a similar sensitivity to methacholine and baseline FEV_1% predicted and comparable number of pack-years. Joyce and coworkers (25) have also reported that in their subjects with airways hyperresponsiveness to histamine smokers were more sensitive to AMP than were ex-smokers. In contrast, our healthy smoking subjects without airways hyperresponsiveness to methacholine were not sensitive to AMP. The lower geometric mean PC_{20} AMP value of the smoking COPD group was similar to the value of the nonsmoking asthmatics. This finding corresponds with results reported by Pin and coworkers (19). Expressed as AMP/methacholine ratio, however, AMP was significantly less potent in the smoking COPD group as compared to the nonsmoking asthmatics. Smoking is a known determinant of the FEV_1, whereas the airway diameter in turn determines the airways responsiveness to methacholine in COPD (26). In addition, this study shows that in subjects with hyperresponsiveness to methacholine, smoking makes them more susceptible to airways narrowing upon AMP inhalation. We could not detect a correlation between the pack-years of smoking or number of cigarettes smoked per day and the level of AMP responsiveness. This suggests that inflammatory changes in the airways elicited by smoking determine the AMP sensitivity in subjects with COPD.

AMP is a stimulus 'indirectly' causing airways constriction. Inhaled AMP is rapidly dephosphorylated to adenosine, which probably acts by stimulation of purinoceptors on cell surfaces (27). Exactly which cells are involved is as yet uncertain. As adenosine has a short half-life time, Ng and coworkers (27) suggested that a superficial effect at the level of epithelial tissues is a more likely target than the

Figure 3. Relations and regression lines of $\log_2 PC_{20}$ methacholine (*open circles*) and $\log_2 PC_{20}$ AMP (*closed circles*) on FEV_1% predicted in smoking control subjects, smoking subjects with COPD, nonsmoking subjects with COPD and nonsmoking asthmatics. Dashed line indicates highest concentrations administered. For methacholine, the equations of the regression lines are (COPD sm): $y = 0.06x - 2.0$, (COPD nsm): $y = 0.04x - 0.6$ and (asthma): $y = 0.09 - 6.0$. For AMP, the equations of the regression lines are (COPD sm): $y = 0.10x - 0.1$, (COPD nsm): $y = 0.04x + 5.1$, (asthma) $y = 0.06x - 1.8$.

deeper layer of smooth muscle cells. Indeed *in vitro* studies have shown that adenosine produced only a very weak contraction of human bronchiolar segments (28), indicating that a 'direct' effect on smooth muscle cells is unlikely. Therefore, mechanisms which can be proposed for the increased AMP responsiveness in atopic asthmatics, the smoking subjects with COPD, and, to a lesser extent, in the nonsmoking subjects with COPD, include release of bronchoconstrictive mediators by mast cells or other inflammatory cells and action via neural reflex mechanisms.

Mast cell release of histamine and, to a lesser extent prostaglandins has been demonstrated in AMP-induced airways constriction (17, 29). A potent histamine H_1-receptor antagonist inhibited as much as 80% of the airways constrictive response to AMP in atopic and nonatopic asthmatics. *In vitro* studies have shown mast cell release by an A_2-purinoceptor-mediated mechanism (30). Although functional A_2-receptors have been reported on many other cell types, among which eosinophils and neutrophils, no evidence exists that adenosine stimulation evokes release of bronchoconstrictive mediators by these cells. In contrast, it has been demonstrated in many studies that release of superoxide anions by eosinophils and neutrophils is inhibited upon A_2-purinoceptor stimulation (31, 32), rather suggesting a scavenging role for adenosine.

Therefore, if release of bronchoconstrictive mediators by inflammatory cells is the main pathway by which AMP is acting, the results of this study suggest that increased susceptibility of mast cells is not only involved in allergen-mediated inflammatory processes in asthma, but also in COPD and in smoking-induced inflammatory processes. This is in agreement with findings of higher N^{τ}-methyl-histamine levels in the urine of subjects with COPD and increased tryptase and histamine levels in the bronchoalveolar lavage fluids of smokers (13, 14).

Next to inflammatory cells there is some evidence that neural reflex mechanisms may contribute to AMP-induced airways constriction. Involvement of central vagal reflex mechanisms is supported by findings in experimental and human studies that muscarinic antagonists oppose the adenosine-induced airways constriction (16, 33). Lidocaine also reduces the adenosine-induced airways constriction, suggesting involvement of irritant receptors (33). Polosa and coworkers (34) reported a reduced airways constriction upon AMP challenge after induction of bradykinin tachyphylaxia, suggesting involvement of local peptidergic reflex mechanisms.

It is conceivable that, as a consequence of inflammation, exposure of sensory nerves because of epithelial damage leads to enhanced release of bronchoconstrictive

neurotransmitters via axon reflex mechanisms. However, activation of local axon reflexes directly by adenosine or indirectly by mediators released from activated inflammatory cells is mainly speculative.

In conclusion, this study demonstrates that all but two subjects with COPD responded with airways narrowing upon AMP provocation. Patients with COPD were significantly more responsive to AMP and methacholine than healthy smoking volunteers. In smoking subjects with COPD and nonsmoking atopic asthmatics, the AMP sensitivity was significantly higher than in nonsmoking subjects with COPD, whereas the sensitivity to methacholine was similar in the asthma and COPD groups.

The results indicate that additional factors other than direct smooth muscle contraction may play a role in both asthma and COPD. Results from other studies have shown that hypersensitivity to allergens is one of the factors that determines the degree of airways responsiveness to AMP in asthma (18). This study has shown that smoking determines the degree of airways responsiveness to AMP in subjects with COPD. This increased sensitivity to AMP provocation suggests an increased susceptibility of mediator release by mast cells or neural reflex mechanisms in asthma and (smoking) subjects with COPD.

Acknowledgements

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Chapter 10

AIRWAYS RESPONSIVENESS TO ADENOSINE 5' - MONOPHOSPHATE IN SMOKERS AND NONSMOKERS WITH ATOPIC ASTHMA

Ytske Oosterhoff, Maarten A.M. Jansen, Dirkje S. Postma, Gerard H. Koëter

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Abstract

Inhalation of adenosine 5'- monophosphate (AMP) has been demonstrated to induce airways constriction indirectly via inflammatory cells or neural reflex mechanisms. Smoking has been shown to determine the AMP sensitivity in subjects with chronic obstructive pulmonary disease. In this study the additional influence of smoking on airways responsiveness to AMP was investigated. AMP and methacholine inhalation provocation tests were performed in smokers and nonsmokers with atopic asthma and nonatopic control subjects who smoke.

Geometric mean PC_{20} AMP was significantly lower in nonsmokers with asthma than in smokers with asthma (3.8 and 15.1 mg/ml, respectively; $p = 0.02$), representing a difference of 2.0 doubling concentrations, whereas PC_{20} methacholine (0.56 and 1.0 mg/ml, respectively), baseline FEV_1 and the atopic state were comparable. Both groups of subjects with asthma were significantly more responsive to AMP and methacholine than the control group (PC_{20} AMP: 357 mg/ml and PC_{20} methacholine: 123 mg/ml; $p < 0.001$).

As the only significant difference in clinical characteristics between the nonsmokers and smokers with asthma was the use of therapy to reduce symptoms, the results suggest that airways responsiveness to AMP is a more sensitive measurement of disease activity than airways responsiveness to methacholine in subjects with atopic asthma.

Introduction

Airways hyperresponsiveness is a characteristic feature in subjects with asthma. The severity of airways responsiveness can be assessed with a variety of inhaled stimuli. Inhalation of adenosine 5'- monophosphate (AMP) has been demonstrated to induce airways constriction indirectly via stimulation of purinoceptors on inflammatory cells, notably mast cells, or via neural reflex mechanisms (1, 2), whereas methacholine is a stimulus that induces airways constriction mainly by direct stimulation of the muscarinic receptors on airways smooth muscle cells. Hypersensitivity to allergens has been shown to determine the degree of AMP airways responsiveness in atopic subjects, because it has been demonstrated that allergen exposure temporarily increases AMP airways responsiveness (3). Because it has been found as well that the sensitivity to AMP in subjects with nonatopic asthma was increased to the same degree as in those with atopic asthma (4), it is likely that other factors are involved in

determining the degree of airways responsiveness to AMP.

Recently, we found that smoking was associated with increased airways responsiveness to AMP in nonatopic subjects with chronic obstructive pulmonary disease. This suggests that inflammatory changes in the airways elicited by smoking determine the AMP sensitivity in chronic obstructive pulmonary disease (5). In this study we investigated the additional influence of smoking on airways responsiveness to AMP in atopic asthma.

Methods

Sixteen nonsmokers and 12 smokers with atopic asthma and 5 nonatopic healthy smokers, aged 18 to 45 years, were selected on basis of previously described criteria (5). The study was approved by the hospital medical ethics committee, and written informed consent was obtained before the start of the study.

From four weeks before the start of the study, subjects were allowed to use inhaled bronchodilators only. Treatment with inhaled corticosteroids, cromoglycate and theophylline was stopped. Inhaled bronchodilators, if used, were withdrawn at least 8 hours before each provocation test.

Subjects attended the laboratory on two visits at the same time of day. On the first day a methacholine provocation test was performed, which was followed, after 2 to 7 days, by an AMP provocation test. Inhalation provocation tests were performed with doubling concentrations of 0.03 to 256 mg/ml (0.15 - 1308 mmol/L) methacholine chloride or 0.04 to 320 mg/ml (0.11 - 913 mmol/L) AMP (Sigma Chemical Co., St Louis, Mo.), according to a 2-minute tidal breathing method (5). The challenge was discontinued when forced expiratory volume (FEV_1) had fallen by 20 % or more of the prechallenge level or when the highest concentration had been administered. Provocative concentrations causing a 20% fall in FEV_1 (PC_{20}) values were calculated by linear interpolation between the last two data points of the logarithmic concentration-response curve. If at the highest concentration administered FEV_1 had fallen < 20% of the prechallenge level, the doubling concentration of the highest concentration was used as the PC_{20} value; if FEV_1 had fallen more than 15% at the highest concentration administered, the PC_{20} value was calculated by linear extrapolation of the logarithmic concentration-response curve.

PC_{20} values were analyzed after base 2 logarithmic tranformation. After verification of normal distributions with the Kolmogorov-Smirnov goodness of fit test, differences

in parameters between groups were analyzed by using Student *t* test for unpaired observations. Nonnormal distributed variables were compared with Mann-Whitney U tests. A two-tailed *p* value of less than 0.05 was considered significant. Data are presented as means (±SD). All analyses were performed with the SPSS/PC+ V4.01 software package (SPSS Inc., Chicago, Ill.).

Results

Subject characteristics are listed in table 1. Between the smokers and nonsmokers with asthma no significant difference was found in mean values of age, baseline FEV_1 % predicted and number of positive skin test results. The only difference was medication use, which was larger in the nonsmoking group. All 16 nonsmokers with asthma used inhaled bronchodilators ($ß_2$-adrenergics or ipratropium bromide), whereas only 6 of the 12 smokers with asthma needed bronchodilator therapy ($p = 0.002$). Besides, 11 of the 16 nonsmokers with asthma used inhaled corticosteroids or cromolyns, in contrast to only 2 of the 12 smokers with asthma ($p = 0.007$).

Percent predicted FEV_1 values were higher in the control subjects as compared with both groups of subjects with asthma, reaching significance with the nonsmokers with asthma ($p=0.02$). The number of pack years smoked was significantly lower in the control group as compared with the group of smokers with asthma ($p=0.045$), but the

Table 1. Subject characteristics. Means ± SD.

	Nonsmokers with asthma	Smokers with asthma	Control smokers
Number	16	12	5
Age (yr)	28.8 ± 7.1	25.0 ± 4.3	24.0 ± 3.3
Medication use	16/16	6/12[1]	0
Atopy[2]	4.7 ± 2.8	5.8 ± 2.4	0
Pack years	NA	5.2 ± 2.4	2.3 ± 1.4[3]
No. of cigarettes	0	15.8 ± 5.8	17.6 ± 8.1
FEV_1 (% predicted)	87.6 ± 3.4	90.8 ± 16.2	102.9 ± 9.3[4]

NA = not assessed;
1 $p = 0.002$ versus nonsmokers with asthma;
2 Number of positive skin tests;
3 $p = 0.02$ versus smokers with asthma;
4 $p = 0.02$ versus nonsmokers with asthma.

mean daily number of cigarettes used was not different.

Mean baseline percent predicted FEV_1 values were not significantly different within the three groups before the two different provocation tests. Geometric mean PC_{20} methacholine values did not differ significantly between the two groups with asthma (Figure 1). The geometric mean value in the nonsmoking group was 0.56 mg/ml and in the smoking group 1.0 mg/ml, representing a difference of 0.8 doubling concentrations. The geometric mean PC_{20} value in the smoking control group was significantly higher as compared with both groups of subjects with asthma (122.8 mg/ml; $p<0.001$). Two subjects in the control group did not respond when the highest concentration of methacholine (256 mg/ml) was administered. The geometric mean PC_{20} AMP was significantly higher in the smokers with asthma than in nonsmokers with asthma (15.1 and 3.8 mg/ml, respectively, $p=0.02$)(Figure 1), representing a difference of 2.0 doubling concentrations. The geometric mean PC_{20} AMP value of the control group was significantly higher compared with both groups of subjects with asthma (357 mg/ml; $p<0.001$). Three subjects in the control group did not respond when the highest concentration of AMP was administered (320 mg/ml).

Discussion

This study demonstrates a significantly higher sensitivity to AMP provocation in nonsmokers with asthma as compared with smokers with asthma, whereas the baseline FEV_1, the sensitivity to methacholine provocation and the atopic state were comparable between the groups. However, the use of therapy to reduce symptoms was remarkably higher in the group of nonsmokers with asthma as compared with the group of smokers with asthma. This suggests that inflammatory factors related to disease activity rather than smoking determine the PC_{20} AMP in asthma.

In contrast to findings in this study in young subjects with asthma, smoking has been found to determine the AMP sensitivity in elderly subjects with increased methacholine or histamine airways responsiveness. AMP airways responsiveness was found to be higher in smokers than in nonsmokers with chronic obstructive pulmonary disease, along with comparable values of baseline FEV_1 and sensitivity to methacholine provocation (5). Correspondingly, preliminary results from a study by Joyce et al. (6) showed that among subjects with airways hyperresponsiveness to histamine, current smokers were more sensitive to AMP provocation than former smokers. Smoking itself did not induce AMP airways responsiveness, because healthy smokers without airways

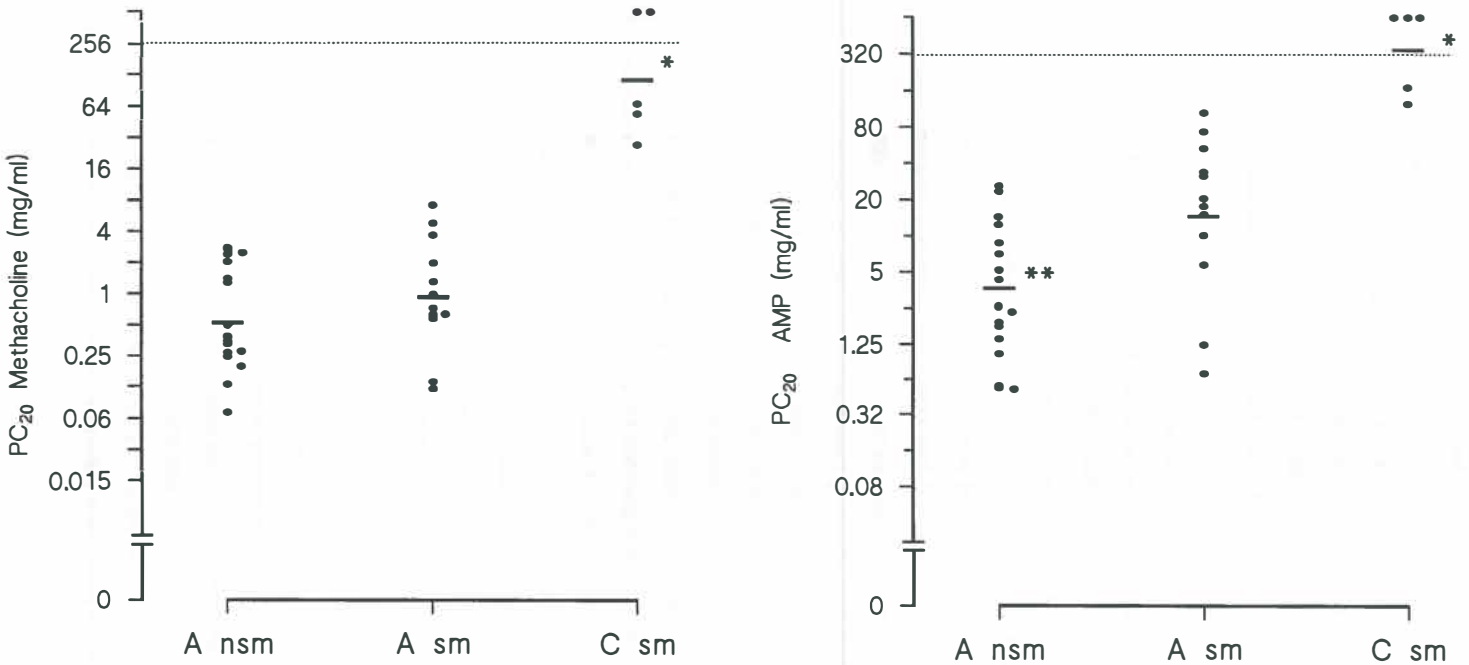

Figure 1. PC_{20} methacholine (*left*) and PC_{20} AMP (*right*) values of nonsmokers with atopic asthma (*A nsm*), smokers with atopic asthma (*A sm*) and nonatopic control subjects who smoke (*C sm*). One unit in PC_{20} represents one doubling dilution. Individual values are represented by dots. Mean values, expressed as geometric mean, are represented by horizontal bars. The dashed line indicates the highest concentration administered. * $p < 0.001$ compared with both groups of subjects with asthma; ** $p = 0.02$ compared with the group of smokers with asthma.

responsiveness to methacholine were not sensitive to AMP provocation (5), which confirms findings in this study. Results of this study do not exclude that smoking is of influence on airways responsiveness to AMP in young atopic subjects with asthma; however, other factors seem to contribute to the airways responsiveness to AMP to a larger extent. Conversely, it can be considered that the results of this study show that smoking decreased sensitivity to AMP (e.g. by increased degranulation of mast cells in individuals who smoke).

The habit of smoking itself may have influenced our patient selection and airways responsiveness to methacholine. Firstly, a so called 'healthy smoker effect' may be present. It has previously been reported that individuals with severe asthma more frequently experience bronchial obstruction on exposure to cigarette smoke (7). As a consequence, subjects with more severe disease will more often refrain from smoking. This may have influenced the selection of subjects in the different groups in our study. Second, in subjects with allergic rhinitis it has been demonstrated that smoking and atopy have a combined effect to increase airways responsiveness to methacholine (8). It must therefore be realized that smoking may have increased the degree of methacholine responsiveness in our smokers with atopic asthma, resulting in a relatively lower PC_{20} value. This may explain why the PC_{20} methacholine values in both groups of subjects with asthma are comparable.

The discrepancy in AMP sensitivity between the groups of smokers and nonsmokers with asthma cannot be explained by differences in therapy used, as the antiinflammatory therapy had been discontinued for 4 weeks before the study. Antiinflammatory therapy is known to reduce AMP sensitivity to a great extent (9). However, the nonsmokers with asthma used more antiinflammatory therapy as maintenance treatment than the smokers with asthma. Thus results would have been opposite if therapy still influenced airways responsiveness 4 weeks after discontinuation.

In conclusion, this study demonstrates that in contrast to findings in elderly subjects with increased airways responsiveness to methacholine, smoking does not increase sensitivity to AMP in young subjects with atopic asthma. The nonsmokers with atopic asthma had significantly lower airways responsiveness to AMP as compared with the smokers with atopic asthma, along with comparable FEV_1, airways responsiveness to methacholine and atopy, as reflected in the number of positive skin test results. Because the only difference between the nonsmokers and smokers with

asthma was the use of therapy to reduce symptoms, the results suggest that airways responsiveness to AMP is a more sensitive measurement of disease activity than airways responsiveness to methacholine in subjects with atopic asthma.

Acknowledgements

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Chapter 11

SUMMARY AND DISCUSSION

11.1 Summary

The studies described in the first part of the thesis were aimed to give more insight in the contribution of inflammatory changes in the occurrrence of nocturnal asthma. In the second part of the thesis the surplus value of measurement of indirect airways responsiveness upon stimulation with AMP was investigated and compared with direct airways responsiveness to methacholine in smoking and nonsmoking subjects with COPD and asthma.

Chapter 2 contains a literature study on the relationship between inflammation and airways hyperresponsiveness in asthma. It is brought out that as yet no provocative stimulus exists that can be used as a parameter of specific inflammatory changes in the airways. Relationships with methacholine- or histamine-induced airways responsiveness have been found for various inflammatory parameters, but not consistently. Therefore, a lance is broken for future evaluation of relationships between airways responsiveness to indirect stimuli and specific inflammatory changes in the airways.

In the study described in *chapter 3* we have investigated whether the occurrence of nocturnal asthma was more associated with an increased (day-night change in) airways responsiveness to the indirect stimuli AMP and propranolol than to direct stimulation with methacholine. Inhalation provocation tests were performed at 16.00 h and at the following night at 04.00 h in nonsmoking atopic asthmatic subjects with and without increased nocturnal airways obstruction. It was found that a more severe airways responsiveness to methacholine as well as AMP and propranolol at daytime was significantly associated with a larger circadian PEF-rhythm in atopic asthmatic subjects. A higher correlation was observed for the indirect stimuli, suggesting that the occurrence of a larger circadian variation in airway diameter is associated with an increased susceptibility to stimulation via indirect mechanisms. Besides, we found a highly significant and positive correlation between the circadian PEF-rhythm and the (16.00 - 04.00 h) change in airways responsiveness to AMP, but not to the other stimuli, whereas the PC_{20} AMP decreased more than PC_{20} methacholine during the night in the group with increased circadian PEF-rhythm. These findings suggest that indirect mechanisms are involved in the development of nocturnal airways constriction which are specific for AMP, as PC_{20} propranonol did not show day-night changes. If mast cell release is the main pathway by which AMP exerts its effect, the results favour a role for mast cell mediator release in the occurrence of nocturnal airways constriction in asthma.

In the second study (*chapter 4*) the effect of propranolol inhalation-provocation tests on the diurnal increase in FEV_1 and propranolol airways hyperresponsiveness was investigated. Propranolol inhalation-provocation tests were performed at one day at 8.00 and 16.00 h, another day at 16.00 h in atopic subjects with asthma. Propranolol inhalation at 8.00 h opposed the diurnal increase in FEV_1. The PC_{20} propranolol values at 8.00 and 16.00 were not different. The results indirectly suggest a role for circulating adrenaline in the diurnal modulation of the airway tone. Besides, propranolol inhalation challenge tests are a less suitable parameter for repeated measurement of the indirect airways responsiveness within one day. Other indirectly acting stimuli are recommended.

In the two following studies (*chapters 5 and 6*) it was investigated whether the occurrence of increased nocturnal airways narrowing was associated with increased (day-night changes in) inflammatory cell numbers and activation in BAL-fluid and peripheral blood samples. Material was obtained at 16.00 and 04.00 h, with an interval of one week and in a randomized order, from atopic asthmatic subjects with and without increased nocturnal airways obstruction and from nonatopic healthy subjects. Increased nocturnal airways obnstruction was on the whole neither associated with day-night changes in cell number nor with cell activation in BAL-fluid and blood. At daytime, however, various cellular differences were found that distinguished the asthma group with increased nocturnal airways narrowing from the one without nocturnal airways narrowing. The level of serum ECP, a mediator released by activated eosinophils, was increased, as was the level of prostaglandin D_2 in BAL-fluid, a mediator mainly derived from activated mast cells. Increased expression of the complement receptor CD11b on alveolar macrophages was found, indicating activation of these cells. Furthermore, lower numbers of BAL $CD4^+$ lymphocytes were found, with lower expression of HLA-DR on the surface at night. This suggests that these cells lag behind in the airway submucosa in subjects with nocturnal asthma, where they may be activated or activate other cells themselves by production of cytokines.

In the following study (*chapter 7*) it was investigated whether dag-night changes occurred in inflammatory cells in the bronchial submucosa from atopic subjects with asthma and nonatopic healthy subjects. The numbers of $CD4^+$ cells at night were higher than at daytime in the biopsies from the asthmatic subjects, but not in the healthy subjects. Further research is needed to investigate whether differences exist between asthmatic subjects with and without nocturnal asthma.

From the results of above described studies it appears that the occurrence of increased nocturnal airways narrowing is related to the enhanced airways responsiveness to indirect stimuli and inflammatory cell activation at daytime rather than increased inflammatory cell activation at night. This led us to question whether nocturnal asthma has to be regarded as a unique entity or merely as a manifestation of more severe asthma. Arguments pro and contra, based on our own studies and other research groups, have been reviewed in *chapter 8*. Based on these arguments, we propose to consider nocturnal asthma as a manifestation of more severe illness and to change the term nocturnal asthma in 'asthma with nocturnal symptoms'.

In the study described in *chapter 9* the inflammatory contribution to airways narrowing in COPD was investigated by assessment of the airways responsiveness upon stimulation with AMP. AMP caused airways narrowing in all but two of the 30 nonatopic subjects with COPD. However, they responded less sensitive to AMP when compared to a group of atopic subjects with asthma with a similar degree of airways responsiveness to methacholine. Furthermore, it was found that the airways responsiveness to AMP was significantly lower in the smokers as compared to the nonsmokers with COPD. As AMP-induced airways constriction is thought to occur via mediator release by mast cells or neural reflex mechanisms, the results suggest that these pathways are involved in the development of airways constriction in COPD, whereas inflammatory changes elicited by smoking determine the degree of airways responsiveness to AMP.

In addition, in *chapter 10* the effect of smoking on the airways responsiveness to AMP was investigated in atopic subjects with asthma. Initially to our surprise, a significantly lower PC_{20} AMP was found in the nonsmokers when compared with smokers, while the baseline FEV_1, PC_{20} methacholine and atopic state were comparable between the groups. A so called 'healthy smoker effect' may have influenced patient selection. With this term is meant that subjects with more severe asthma will sooner refrain from smoking, experiencing dyspnea as the consequence of smoking-induced airways constriction. This phenomenon was supported by the finding that the use of drug therapy to reduce asthma symptoms was remarkably higher in the nonsmokers than in the smokers with asthma. The results, therefore, suggest that measurement of the PC_{20} AMP is a more sensitive parameter of disease activity than the PC_{20} methacholine in atopic subjects with asthma.

11.2 Discussion

11.2.1 Inflammation in nocturnal asthma

The studies described in this thesis confirm the hypothesis that inflammation contributes to the occurrence of nocturnal asthma. Although indirect evidence exists for increased nocturnal inflammation, generally the cellular data indicate that nocturnal asthma is more likely to occur in asthmatic subjects with signs of increased inflammatory cellular activation in the airways at daytime. In this respect, nocturnal asthma can be regarded as a manifestation of more severe illness.

From the results it can also be concluded that different inflammatory cell types may be involved and that not one single cell type, activation product or inflammatory pathway can be assigned as principal in the pathogenesis of nocturnal asthma, as assessed by measurement of indirect airways responsiveness and determination of numbers and activation of different specific cell subtypes that are known to be related with airways narrowing or airways responsiveness in atopic asthma. Several causes can be mentioned for not finding specific inflammatory activation in our subjects with nocturnal asthma. Firstly, changes may only occur in asthmatic subjects with more severe nocturnal asthma. As compared with other research groups (1-3) we selected a somewhat milder group of asthmatic subjects, as a consequence of the inclusion criterium that the participants had to refrain from anti-inflammatory drugs from four weeks before the start of the study, in order to prevent confounding of the results by anti-inflammatory treatment. This resulted in a drop out of at least one third of our candidates during the 'wash-out' period, because of a too severe flaring up of their asthma symptoms. Secondly, specific inflammatory changes may occur principally in the airway submucosa. Investigation of changes in peripheral blood and BAL-fluid may therefore not optimally reflect local bronchial changes. This is suggested by findings in the atopic subjects with asthma, showing a significant increase of $CD4^+$ cells at night in the bronchial biopsy specimens without day-night changes in the BAL $CD4^+$ cell numbers. Therefore, more research is needed to investigate local bronchial inflammatory changes at night in asthmatic subjects with and without increased nocturnal airways constriction. Furthermore, more insight needs to be obtained whether differences between healthy and asthmatic subjects exist in circadian changes of cellular traffic between the different compartments (bone marrow, peripheral blood, bronchial submucosa and bronchoalveolar space). Thirdly, the assessment of inflammatory activity is limited by the sensitivity and the specifity of the parameters

used. For example, assessment of inflammatory changes by measurement of airways responsiveness to AMP is a convenient technique, however of limited value to give an exact judgement on the specific inflammatory pathway involved. In contrast, assessment of ECP, a specific marker of eosinophil activation, was limited by too low detection values in the BAL-fluid. For the same reason, it was until now impossible to assess cytokines in BAL-fluid that are known to be involved in inflammatory processes in asthma (*e.g.* GM-CSF, IL4 and IL5). Refinement of laboratory techniques will extend the possibilities to determine more specific activation markers in the near future. Furthermore, investigating different cellular parameters, the spread of the measured values of different parameters within the groups was often larger than expected as based on findings of comparable previously published studies (4, 5). Therefore, differences may have not be found as a consequence of too small group sizes. Finally, it can be hypothesized that specific inflammatory changes may not primarily underlie nocturnal asthma. As such, inflammatory processes in nocturnal asthma are similar to those in asthma in general, characterized by specific inflammatory activation (eosinophils and T_{H2}-lymphocytes), inducing pathologic changes in the airways and distinguishing asthmatic from healthy individuals. In addition, many more cells and cellular networks can be involved in the pathologic mechanisms that underlie or enhance the inflammatory process in asthma.

The reason why nocturnal asthma nevertheless has to be distinguished as a research area of special interest and concern is that especially at night the airway tone is prominently influenced by chronobiological circadian rhythms (figure 1). It is likely that circadian rhythms modulate the cholinergic and nonadrenergic-noncholinergic (NANC) neurotransmission, increasing the sensitivity of the airway smooth muscle tone at night to inflammatory and other triggers. Hereby, the lower adrenaline and cortisol levels at night may play an important role, whereas exogenous factors that have been proposed to explain nocturnal asthma (6) particularly increase the supply of inflammatory mediators or directly trigger the 'primed' autonomic system.

Results from studies on adrenaline have made clear that circulating adrenaline probably does not exert a direct effect on airway smooth muscle itself. This was supported by findings that no improvement of the nocturnal fall in lung function was found upon correction of the nocturnal fall in adrenaline with adrenaline infusion in subjects with nocturnal asthma (7). Furthermore, after bilateral adrenalectomy a

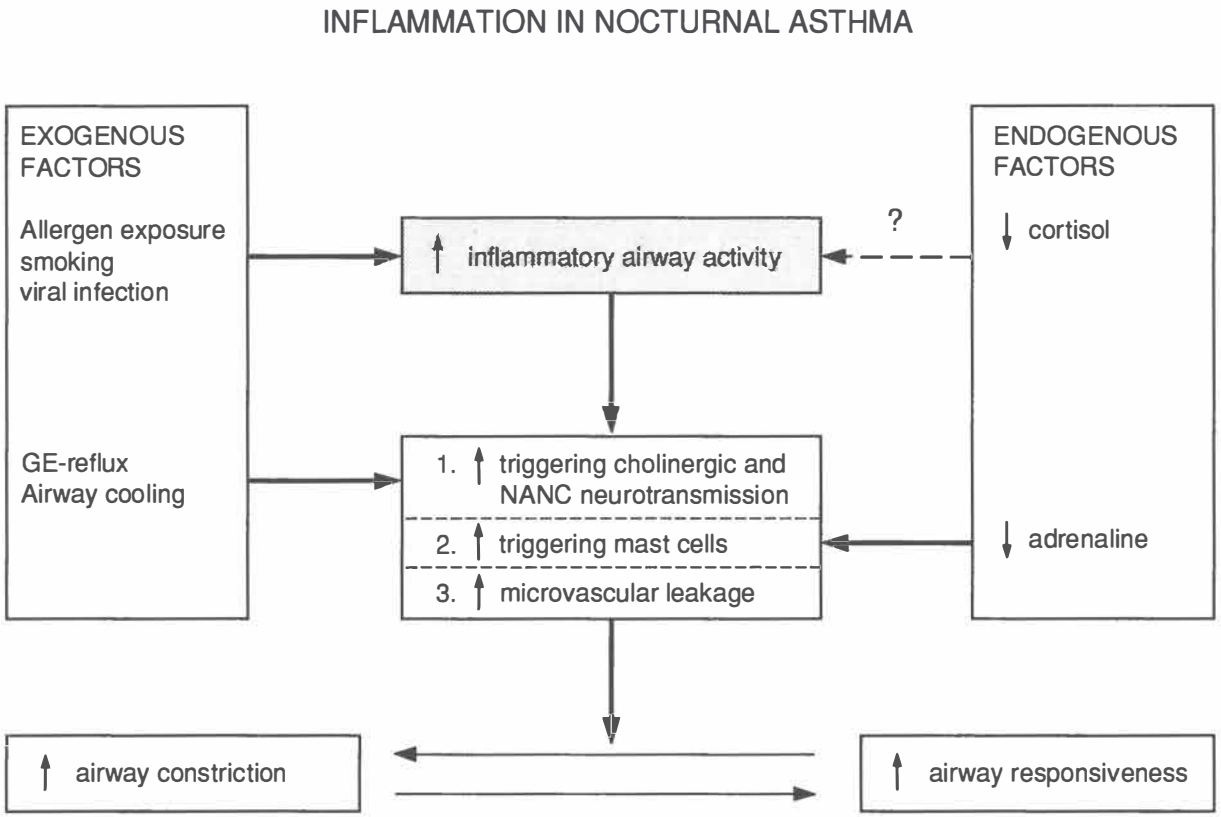

Figure 1. Hypothetical model of factors contributing to the occurrence of nocturnal asthma.

circadian variation in lung function still existed (8). However, circulating adrenaline may indirectly influence the nocturnal airway diameter, as supported by our finding that inhalation of the ß-blocker propranolol at 8:00 AM opposed diurnal increase in FEV_1. Lower adrenaline levels at night may oppose the inhibitory effect on release of histamine and other mediators by ß-adrenergic stimulation of mast cells, influence cholinergic tone by opposed inhibition of cholinergic and NANC neurotransmission and oppose the prevention of microvascular leakage in the airways (9, 10).

Involvement of the cholinergic neurotransmission in the occurrence of nocturnal asthma is supported by the demonstration that intravenous infusion of atropine, resulting in adequate cholinergic blockade, substantially reduced the nocturnal airways constriction in asthmatic adults (11). In this respect, an increased peripheral cholinergic sensitivity is probably more important than increased central vagal activity. Conflicting results have been found in studies investigating changes in the circadian variation in central vagal activity in children and adults with asthma and chronic airflow limitation, as determined by heart rate and the sinus arrhythmia gap (12-15). Furthermore, after heart-lungtransplantation, when central vagal innervation is lost, a diurnal rhythm in airway diameter still exists (16). As a consequence of an opposed inhibition of cholinergic neurotransmission by decreased levels of circulating adrenaline, the sensitivity of this pathway to inflammatory and other triggers may be increased. Increased local cholinergic activity can be induced by an enhanced efferent stimulation, for instance because inflammatory mediators antagonize the negative feedback on the neurotransmission via M_2-receptors, and possibly other suppressing receptors (17). In addition, increased afferent stimulation via the sensory nerves can be effectuated by inflammatory and other triggers.

The NANC-tone also shows a circadian variation (19). In contrast to subjects without nocturnal asthma, it was found that in nocturnal asthmatics no circadian variation existed in response to capsaicin, suggesting that the NANC-neurotransmission is impaired (20). It has been reviewed that inflammatory products can influence NANC tone in a number of ways (21). The exact contribution of this system to the occurrence of nocturnal asthma has yet to be determined, *e.g.* by means of specific blockers of the NANC-neurotransmission.

Less evidence exists about a modulatory role of the nocturnal fall in circulating cortisol on the occurrence of nocturnal asthma. A direct effect of endogenous cortisol on the airway diameter is unlikely, as demonstrated by findings that correction of the

nocturnal fall in cortisol levels by intravenous physiologic doses of hydrocortisone did not totally abolish the nocturnal fall in pulmonary function in five of the six asthmatic participants (22). Nevertheless, as corticosteroids are known to suppress the inflammatory responses in the airways submucosa (23-25), it still cannot be excluded that the midnight fall in circulating cortisol levels results in an intensification of local inflammatory responses in the airways, indirectly exerting a deteriorating effect on the airway diameter at night. More research is needed to elucidate the influence of circadian swings in cortisol levels on inflammatory activity in the airway submucosa.

In clinical practice, with the present knowledge nocturnal asthma can very well be regarded as a manifestation of more severe asthma. Treatment strategies will therefore not primarily be focused on the nocturnal symptom, but on asthma in general. This includes elimination of provocative factors, *e.g.* reduction of allergen exposure in atopic subjects and adequate treatment of a diagnosed gastro-oesophageal reflux. If these measures are not successfull, a profit can be made by reduction of inflammatory activity in the airways prescribing anti-inflammatory drugs. If this is not effective enough, the following step is addition of bronchodilators that act long enough to bridge the sleeping period.

11.2.2 Inflammation and AMP airways hyperresponsiveness

Studies on airways hyperresponsiveness in this thesis have one by one shown that measurement of AMP-induced airways responsiveness gives additional information, above methacholine-induced airways responsiveness, on the pathogenetic mechanisms underlying airways narrowing in nocturnal asthma and on disease activity in COPD and asthma. Therefore, AMP inhalation provocation tests are a valuable and promising parameter in laboratory research for *in vivo* follow-up of inflammatory airways activity and in evaluating the effects of anti-inflammatory drug therapy in asthma and COPD. In this way, AMP inhalation provocation tests have acquired a place in our current research projects during last years. Therefore, in the near future we will be able to investigate the surplus value of this parameter to a larger extent. However, a major point of limitation in applying AMP inhalation provocation tests remains the interpretation of the results, because uncertainty still exists on exactly which pathways are involved in AMP-induced airways constriction. During the last decade a number of studies have been published that favour a role for mast cell release in AMP-induced airways constriction (26, 27). It must nevertheless be kept in mind that other

pathways, like neural reflex mechanisms (28), may be equally involved and have as yet not been revealed to that extent, as a consequence of limited possibilities to investigate these pathways properly. More research is needed to elucidate the pathways in AMP-induced airways constriction.

11.3 Conclusions

The main findings of the studies described in this thesis are:

1. Relationships with methacholine- or histamine-induced airways responsiveness have been reported for various inflammatory parameters, but they are not consistent. At this moment there is no suitable stimulus that can be used as a parameter of specific inflammatory processes in the airways.
   Inflammation and nocturnal asthma
2. The occurrence of an increased nocturnal airways narrowing in atopic subjects with asthma is correlated with an enhanced indirect and direct airways responsiveness at daytime. In addition, it is correlated with the (16.00 - 04.00 h) change in PC_{20} AMP. PC_{20} AMP decreased more than PC_{20} methacholine during the night in asthmatic subjects with increased nocturnal airways narrowing. The findings suggest that inflammatory mechanisms are involved in the occurrence in nocturnal asthma.
3. Propranolol inhalation at 8:00 AM in atopic subjects with asthma opposes the normal diurnal increase in FEV_1, indirectly suggesting a role for circulating adrenaline in the diurnal modulation of the airway diameter.
4. An increased nocturnal airways obstruction in subjects with atopic asthma is not associated with day-night changes in inflammatory cell number and activation in BAL-fluid and peripheral blood. At daytime the asthmatic subjects with increased nocturnal airways obstruction can be distuinguished from those without increased nocturnal airways obstruction by an increased activation of a variety of specific inflammatory cells in BAL-fluid and peripheral blood.
5. In the bronchial submucosa of asthmatic subjects an influx of $CD4^+$ cells was found at night. Further research is needed to investigate differences between asthmatic subjects with and without nocturnal asthma.
6. Nocturnal asthma can be considered as a more severe manifestation of asthma, as concluded from our findings that the occurrence of increased nocturnal airways

narrowing is associated with increased inflammatory activity at daytime rather than increased inflammatory changes at night.

Inflammation and AMP airways hyperresponsiveness

7. AMP inhalation provocation tests generally cause airways narrowing in subjects with COPD, but they respond less sensitive to AMP than asthmatic subjects with a comparable geometric mean PC_{20} methacholine value. These findings suggest that inflammatory pathways play a role in the occurrence of airways constriction in COPD. In addition, inflammatory processes elicited by smoking determine the degree of AMP responsiveness in COPD.
   In contrast, smokers with asthma respond less sensitive to AMP provocation than nonsmokers with asthma. The airways responsiveness to AMP seems a more sensitive parameter of disease activity than the airways responsiveness to methacholine in asthma.

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SAMENVATTING EN BESPREKING

Samenvatting

In dit proefschrift worden een aantal onderzoeken beschreven waarin werd onderzocht of specifieke ontstekingsprocessen een rol spelen bij het optreden van nachtelijke kortademigheid bij astmapatiënten. Astma wordt in dit proefschrift gekenmerkt als een syndroom dat manifest wordt op jongere leeftijd, en dat gekarakteriseerd wordt door aanvallen van kortademigheid en piepen op basis van luchtwegobstruktie, de aanwezigheid van luchtweg-hyperreaktiviteit en het bestaan van allergie. Nachtelijke kortademigheid treedt op bij veel personen met astma en leidt regelmatig tot onderbreking van de slaap rond 4 uur 's nachts als gevolg van een vernauwing van de luchtwegen. De luchtwegdiameter vertoont een fysiologische circadiane (= 24-uurs) schommeling. Het is gebleken dat bij astmapatiënten met nachtelijke kortademigheid een grotere circadiane schommeling van de luchtwegdiameter optreedt dan bij gezonde personen en astmapatiënten die geen nachtelijke luchtwegvernauwing ontwikkelen. Dit verschijnsel wordt 'nachtelijk astma' genoemd. Ondanks verbeterde mogelijkheden van de astma-therapie en intensief wetenschappelijk onderzoek naar nachtelijk astma gedurende de laatste decennia blijft nachtelijke kortademigheid een frequent voorkomend en ernstig symptoom. Het gevolg is ondermeer dat de astma-mortaliteit 's nachts het hoogst is en de cognitieve funkties bij patiënten met nachtelijk astma overdag zijn verminderd. Meer inzicht in het pathofysiologische mechanisme dat aan de toegenomen nachtelijke luchtwegvernauwing ten grondslag ligt zou kunnen leiden tot het ontwikkelen van betere therapeutische strategieën teneinde dit hinderlijke symptoom het hoofd te kunnen bieden.

In het algemeen wordt gedacht dat de nachtelijke luchtwegvernauwing een uiting is van een nachtelijke toegenomen gevoeligheid van de luchtwegen voor allerlei prikkels: luchtweg-hyperreaktiviteit. Het is bekend dat specifieke ontstekingsprocessen in de luchtwegen de luchtweg-hyperreaktiviteit bij astmapatiënten doet toenemen. Dit was aanleiding om te onderzoeken of specifieke inflammatoire veranderingen ten grondslag liggen aan het optreden van nachtelijk astma. Dit werd op twee verschillende wijzen onderzocht. *Indirect* werd informatie over ontstekingsaktiviteit in de luchtwegen verkregen door verschillen in luchtweg-hyperreaktiviteit overdag en 's nachts niet alleen te meten met stimuli die luchtwegvernauwing teweeg brengen door direkte contractie van de gladde spiercel, maar ook stimuli te gebruiken die op indirekte wijze, via stimulatie van ondermeer inflammatoire cellen, gladde spiercelcontraktie induceren. De reaktiviteit van de luchtwegen kan in het longfunktie-laboratorium worden gemeten met

behulp van inhalatie-provokatietesten. Hierbij worden oplopende concentraties van een bepaalde stimulus geïnhaleerd en de respons gemeten met behulp van een longfunktie-parameter, in onze studies de éénsecondecapaciteit, in de Engelse benaming aangeduid als "forced expiratory volume (FEV_1)". De ernst van de hyperreaktiviteit wordt in het algemeen uitgedrukt in die provokatie-concentratie van de stimulus waarbij de FEV_1 20% is gedaald ten opzichte van de uitgangswaarde: PC_{20}. De voordelen van inhalatie-provokatietesten zijn dat ze op een gestandaardiseerde wijze kunnen worden uitgevoerd en weinig belastend zijn voor de deelnemers. Daarnaast werd meer *direkte* informatie verzameld door met behulp van flexibele bronchoskopie materiaal uit de luchtwegen te verzamelen en te onderzoeken op de aanwezigheid van ontstekingscellen en hun aktivatiestaat. De mogelijkheid om op een veilige wijze een flexibele bronchoskopie uit te voeren bij astmapatiënten en voortschrijdende nieuwe ontwikkelingen van immunopathologische technieken hebben de afgelopen jaren een beter inzicht gegeven in de specifieke ontstekingsprocessen in de luchtwegen die bij astma een rol spelen.

Op grond van de aldus verkregen informatie werd onderzocht of er aanwijzingen bestaan voor een verhoogde ontstekingsaktiviteit bij astmapatiënten met een toegenomen nachtelijke luchtwegvernauwing.

Hoofdstuk 2 bevat een literatuurstudie over de relatie tussen ontstekingsaktiviteit in de luchtwegen en de mate van luchtweghyperreaktiviteit in astma. Hierbij komt naar voren dat op dit moment geen geschikte stimulus bestaat die kan worden gebruikt als een parameter voor specifieke inflammatoire processen in de luchtwegen. Een associatie tussen metacholine- en histamine-hyperreaktiviteit met verschillende inflammatoire parameters wordt niet eenduidig gevonden. Mogelijk reflecteert de luchtweg-hyperreaktiviteit gemeten met indirekte stimuli beter de specifieke inflammatoire veranderingen in de luchtwegen. Meer onderzoek is nodig om de relatie tussen deze parameters te evalueren.

In de volgende studie (*hoofdstuk 3*) werd onderzocht of het optreden van een toegenomen nachtelijke luchtwegvernauwing beter geassocieerd was met een toegenomen (dag-nacht verandering van) luchtweg-hyperreaktiviteit gemeten met de indirekte stimuli adenosine 5'-monophosphaat (AMP) en propranolol dan met de direkte stimulus metacholine. Hiertoe werden inhalatie-provokatietesten verricht om 16.00 uur en de daaropvolgende nacht om 4.00 uur bij niet-rokende atopische personen met astma tussen 18 en 45 jaar, van tevoren ingedeeld in een groep met ($n = 7$) en zonder

(n = 9) toegenomen nachtelijke luchtwegvernauwing. Een overdag gemeten ernstiger luchtweg-hyperreaktiviteit voor zowel metacholine, AMP en propranolol was significant gecorreleerd met een grotere circadiane variatie in luchtwegdiameter van alle astmapatiënten. Een hogere correlatie werd evenwel gevonden voor de indirekte stimuli. Ook correleerde de (16.00 - 4.00 uur) verandering in PC_{20} AMP significant en positief met de circadiane variatie in luchtwegdiameter van alle deelnemers, maar dit verband werd niet gezien na metacholine-stimulatie. Het bleek dat 's nachts de PC_{20} AMP meer daalde dan de PC_{20} metacholine in de groep met toegenomen nachtelijke luchtwegvernauwing. De bevindingen suggereren dat indirekte mechanismen betrokken zijn bij het optreden van toegenomen nachtelijke luchtwegvernauwing. Deze zijn specifiek voor AMP, aangezien de PC_{20} propranolol geen dag-nacht veranderingen vertoonde. Hoewel niet geheel bekend is welke mechanismen precies betrokken zijn bij de door AMP geïnduceerde luchtwegvernauwing, zijn er aanwijzingen dat de mestcel hierbij van belang is. De resultaten suggereren derhalve dat een verhoogde prikkelbaarheid van de mestcellen om hun mediatoren uit te storten ten grondslag ligt aan de toegenomen nachtelijke luchtwegvernauwing bij patiënten met astma.

In de volgende studie (*hoofdstuk 4*) werd het effekt onderzocht van propranolol inhalatie-provokatietesten op de diurnale toename van FEV_1 en propranolol luchtweg-hyperreaktiviteit. Hiertoe werden inhalatie-provokatietesten met propranolol verricht op de ene dag om 8.00 uur en 16.00 uur en op een andere dag om 16.00 uur bij 15 atopische personen met astma. Propranolol-inhalatie om 8.00 uur 's ochtends bleek de diurnale stijging van de FEV_1 tegen te gaan. Tussen de PC_{20} propranololwaarden op de verschillende tijdstippen werd geen verschil gemeten. Luchtwegvernauwing door propranolol wordt geïnduceerd door blokkade van ß-adrenerge receptoren. De resultaten suggereren indirekt een rol voor circulerend adrenaline in de circadiane modulatie van de luchtwegdiameter. Daarnaast lijkt propranolol door het langdurige remmende effekt op de luchtwegdiameter een minder geschikte parameter voor herhaald onderzoek van indirekte luchtweg-hyperreaktiviteit op dezelfde dag. In dat geval kunnen beter andere indirekte stimuli worden gebruikt.

In de volgende twee studies (*hoofdstukken 5 en 6*) werd onderzocht of het optreden van een toegenomen nachtelijke luchtwegobstruktie geassocieerd is met verhoogde (dag-nacht veranderingen van) aantallen en aktivatie van ontstekingscellen in de bronchoalveolaire lavage (BAL)-vloeistof en in het perifere bloed. Hiertoe werd in gerandomiseerde volgorde en met een interval van een week materiaal verzameld om

16.00 uur en om 4.00 uur bij 17 deelnemers met atopisch astma, waarvan 10 met en 7 zonder toegenomen nachtelijke luchtwegobstruktie, en bij 8 gezonde niet-atopische personen. Een toegenomen nachtelijke luchtwegobstruktie bleek niet geassocieerd met dag-nacht veranderingen in celaantal, -aktiviteit of inflammatoire mediatoren in BAL-vloeistof en perifeer bloed. Overdag werden echter een aantal specifieke verschillen gevonden waardoor de astmagroep met nachtelijke luchtwegvernauwing kon worden onderscheiden van die zonder nachtelijke luchtwegvernauwing: het serum ECP, een aktivatieprodukt van eosinofielen, was verhoogd, evenals de concentratie van prostaglandine D_2 in de BAL-vloeistof, een mediator vooral afkomstig uit geaktiveerde mestcellen. Daarnaast wijst een verhoogde expressie van de complement receptor CD11b op alveolaire macrofagen op aktivatie van deze cellen. Verder werden lagere aantallen BAL $CD4^+$-lymfocyten aangetroffen, terwijl deze cellen 's nachts minder HLA-DR tot expressie brachten, wat wijst op een lagere aktivatiestaat. Dit zou kunnen betekenen dat $CD4^+$ cellen achterblijven in de bronchiale submucosa van patiënten met nachtelijk astma, alwaar zij geaktiveerd kunnen worden of zelf andere cellen aktiveren door eigen cytokineproduktie.

In de volgende studie (*hoofdstuk 7*) werd onderzocht of dag-nacht veranderingen optreden in celaktiviteit in bronchiale slijmvliesbiopten van 6 atopische astmatische en 8 niet-atopische gezonde deelnemers. Het aantal $CD4^+$ cellen was 's nachts hoger dan overdag in de biopten van de astmatici, maar niet bij gezonden. Nader onderzoek is nodig om te inventariseren of er verschillen bestaan tussen astmapatiënten met en zonder toegenomen nachtelijke luchtwegvernauwing.

Uit de resultaten van bovenbeschreven studies blijkt dat het optreden van een toegenomen nachtelijke luchtwegobstruktie in grotere mate gerelateerd is aan de overdag gemeten luchtweg-hyperreaktiviteit voor indirekte stimuli en aan celaktivatie dan aan een verhoogde ontstekingsaktiviteit 's nachts. Dit was aanleiding om ons af te vragen of nachtelijk astma dient te worden beschouwd als een unieke entiteit of louter als een uitingsvorm van ernstiger astma. Argumenten voor en tegen, gebaseerd op eigen studies en van andere onderzoeksgroepen, werden besproken in *hoofdstuk 8*. Op grond hiervan stellen we voor om de term nachtelijk astma te veranderen in: 'astma met nachtelijke symptomen.'

In het laatste deel van dit proefschrift worden aanvullend een aantal onderzoeken beschreven waarin de meerwaarde aan informatie over inflammatoire aktiviteit in de

luchtwegen door het meten van de luchtweg-hyperreaktiviteit met de indirekte stimulus AMP werd onderzocht en vergeleken met de door metacholine geïnduceerde luchtwegvernauwing.

In het onderzoek beschreven in *hoofdstuk 9* werden inhalatie-provokatietesten met AMP en metacholine uitgevoerd bij patiënten met chronische obstruktieve luchtwegaandoeningen, in het Engels aangeduid met de term COPD ('Chronic Obstructive Pulmonary Disease'). Dit syndroom wordt manifest op oudere leeftijd, en wordt gekenmerkt door kortademigheid, die continu en/of bij inspanning aanwezig is, door luchtweghyperreaktiviteit voor metacholine, en door de afwezigheid van allergie. Negentien rokende en 11 niet-rokende patiënten met COPD namen deel aan het onderzoek. De resultaten werden vergeleken met die van 12 oudere rokende niet-allergische gezonden en van 16 atopische niet-rokende deelnemers met astma. Inhalatie-provokatie met AMP veroorzaakte luchtwegvernauwing bij 28 van de 30 patiënten met COPD. De mate van AMP-geïnduceerde luchtwegvernauwing was echter minder groot dan in de groep astmatici, terwijl de PC_{20} metacholinewaarden van beide groepen vergelijkbaar waren. Daarnaast werd gevonden dat de PC_{20} AMP-waarde lager was in de rokende dan in de niet-rokende groep. AMP-geïnduceerde luchtwegvernauwing treedt waarschijnlijk op als gevolg van een uitstorting van mediatoren door mestcellen, ofwel via neurale reflexmechanismen. De bevindingen suggereren dan ook dat inflammatoire mechanismen tevens een rol spelen bij het ontstaan van luchtwegvernauwing bij COPD. Daarnaast lijken ontstekingsprocessen veroorzaakt door roken de mate van AMP luchtweg-hyperreaktiviteit bij COPD te bepalen.

Aansluitend werd de PC_{20} AMP gemeten bij 12 rokende atopische deelnemers met astma en werden de resultaten vergeleken met die van de niet-rokende atopische astmapatiënten (*hoofdstuk 10*). In tegenstelling tot de bevindingen bij de patiënten met COPD, vonden we dat de PC_{20} AMP lager was in de niet-rokende groep astmatici, terwijl de FEV_1-uitgangswaarde, de PC_{20} metacholine en de atopische status vergelijkbaar waren in beide groepen. Waarschijnlijk werd het selekteren van de deelnemers beïnvloed door het zogenaamde 'healthy smoker effect'. Met deze term wordt bedoeld dat personen met ernstiger astma zich eerder zullen onthouden van roken, omdat zij kortademig worden als gevolg van door roken veroorzaakte luchtwegvernauwing. Dit fenomeen wordt ondersteund door de bevinding dat het gebruik van medikatie om astmasymptomen te reduceren beduidend hoger was in de

niet-rokende groep in vergelijking met de rokende groep. De bevindingen suggereren derhalve dat het bepalen van de PC_{20} AMP een gevoeliger maat voor ziekteaktiviteit is dan PC_{20} metacholine bij patiënten met atopisch asthma.

Bespreking

1. Inflammatoire processen en nachtelijk astma.

De studies die in dit proefschrift zijn beschreven bevestigen de hypothese dat inflammatoire processen bijdragen aan het optreden van nachtelijk astma. Hoewel indirekt aanwijzingen werden gevonden voor toegenomen nachtelijke ontstekingsaktiviteit, wijzen de meeste data over celaktiviteit erop dat nachtelijk astma met name optreedt bij astmapatiënten bij wie overdag al aanwijzingen bestaan voor een toegenomen celaktiviteit in de luchtwegen. In dit opzicht kan nachtelijk astma worden gezien als een uitingsvorm van ernstiger astma.

Uit de resultaten kan eveneens worden gekonkludeerd dat verschillende ontstekingscellen betrokken waren bij het inflammatoire proces en dat één enkele cel, aktivatieprodukt of inflammatoir mechanisme dus niet verantwoordelijk kan worden gesteld in de pathogenese van nachtelijk astma, voor zover we dit hebben kunnen vaststellen door het meten van de indirekte luchtweg-hyperreaktiviteit, en het bepalen van aantallen en aktivatiestaat van verschillende specifieke celtypen waarvan bekend is dat ze een rol spelen bij de inductie van luchtwegvernauwing of hyperreaktiviteit in atopisch astma.

Een aantal oorzaken kan worden genoemd waarom wij geen specifieke toename van inflammatoire celaktiviteit vonden in ons onderzoek. In de eerste plaats is het mogelijk dat een nachtelijke recrutering van cellen naar de luchtwegen pas optreedt bij patiënten met een ernstiger toename van de nachtelijke luchtwegvernauwing. Vergeleken met andere onderzoeksgroepen selekteerden wij patiënten met minder ernstig astma, als gevolg van het inklusiekriterium dat de deelnemers gedurende 4 weken voorafgaand aan het onderzoek geen anti-inflammatoire medicijnen mochten gebruiken, omdat deze de resultaten van het onderzoek kunnen beïnvloeden. Als gevolg hiervan viel 1/3 van de kandidaten uit gedurende de 'uitwas'- periode, doordat de astmasymptomen te ernstig opvlamden. Ten tweede bestaat de mogelijkheid dat specifieke inflammatoire veranderingen voornamelijk in het bronchusslijmvlies optreden, zodat onderzoek van veranderingen in perifeer bloed en BAL-vloeistof niet optimaal deze lokale bronchiale veranderingen weerspiegelen. Dit wordt gesuggereerd door

bevindingen in het bronchusslijmvlies van atopische personen met astma, waarin de $CD4^+$ cellen 's nachts significant toenamen, terwijl geen dag-nacht verandering van deze cellen werd gevonden in de BAL-vloeistof. Dit impliceert dat meer onderzoek nodig is naar lokale bronchiale inflammatoire veranderingen bij astmapatiënten met en zonder toegenomen nachtelijke luchtwegobstruktie. Daarnaast dient meer inzicht te worden verkregen of tussen astmapatiënten en gezonden verschillen bestaan in circadiane veranderingen van cellulair verkeer tussen de verschillende kompartimenten (beenmerg, perifeer bloed, bronchusslijmvlies en bronchoalveolaire ruimte). Ten derde wordt het bepalen van inflammatoire aktiviteit beperkt door de mate van sensitiviteit en specificiteit van de onderzochte parameters. Het uitvoeren van AMP luchtweg-hyperreaktiviteit kan bijvoorbeeld eenvoudig en gestandaardiseerd worden uitgevoerd, maar is van beperkte waarde om een uitspraak te doen over de betrokkenheid van een specifiek inflammatoir mechanisme. Daarentegen heeft het bepalen van ECP, een specifiek aktivatieprodukt van eosinofielen, zijn beperking doordat de gemeten waarden in de BAL-vloeistof voornamelijk onder de detectie-limiet lagen. Om dezelfde reden was het tot nu toe niet mogelijk om cytokines te bepalen die betrokken zijn bij het inflammatoire proces in astma (bijv. GM-CSF, IL4 en IL5). Door verfijning van laboratoriumtechnieken zal het in de toekomst mogelijk zijn om meer specifieke aktivatieprodukten te bepalen. Ook viel op dat de spreiding van verschillende gemeten waarden van cellulaire parameters vaak groter was dan verwacht op basis van literatuurbevindingen van vergelijkbaar onderzoek. Verschillen kunnen derhalve niet zijn aangetoond als gevolg van de beperkte groepsgrootte. Tenslotte stellen we de hypothese dat specifieke inflammatoire veranderingen niet primair ten grondslag liggen aan nachtelijk astma. Als zodanig zijn inflammatoire processen in nachtelijk astma vergelijkbaar met astma in het algemeen, gekarakteriseerd door specifieke inflammatoire aktivatie (mestcellen, eosinofielen en T_{H2}-lymfocyten), waardoor pathologische veranderingen in de luchtwegen optreden en astmatische van gezonde personen worden onderscheiden. Daarnaast kunnen vele andere cellen en cellulaire netwerken betrokken zijn bij het pathologische mechanisme, danwel het ontstekingsproces in astma doen versterken.

Desondanks moet nachtelijk astma worden onderscheiden als een onderzoeks-entiteit die speciale aandacht en zorg behoeft, omdat juist 's nachts de luchtwegdiameter prominent wordt beïnvloed door chronobiologische circadiane ritmes (Hoofdstuk 11;

figuur 1). Het is aannemelijk dat circadiane ritmes de cholinerge en nonadrenerge-noncholinerge (NANC) neurotransmissie moduleren, waardoor de luchtwegspiertonus 's nachts gevoeliger is voor inflammatoire en andere prikkels. Lagere circulerende adrenaline- en cortisol-spiegels 's nachts spelen hierbij waarschijnlijk een belangrijke rol, terwijl exogene faktoren met name het aanbod van inflammatoire mediatoren verhogen of direkt het verhoogd prikkelbare autonome systeem stimuleren.

Uit verschillende onderzoeken is gebleken dat het circulerende adrenaline waarschijnlijk geen direkt effekt op het gladde spierweefsel zelf uitoefent. Dit wordt ondersteund door de bevinding dat de nachtelijke daling van de longfunktie niet verbeterde na correctie van de dalende adrenalinespiegel door intraveneuze toediening van adrenaline bij patiënten met nachtelijk astma. Ook bleek na bilaterale adrenalectomie nog steeds een circadiane variatie in longfunktie te bestaan. Het is daarentegen waarschijnlijk dat het circulerende adrenaline indirekt de luchtwegdiameter beïnvloedt, hetgeen wordt ondersteund door onze bevinding dat inhalatie van de ß-blokker propranolol om 8 uur 's ochtends de diurnale toename van FEV_1 opponeerde. Een lagere adrenalinespiegel 's nachts kan het remmende effekt van adrenaline op de uitstorting van histamine en andere mediatoren via ß-adrenerge stimulatie van mestcellen opheffen, de cholinerge tonus beïnvloeden via een opgeheven remming van de cholinerge neurotransmissie, de preventie van microvaskulaire lekkage in de luchtwegen opheffen, terwijl resultaten in een caviamodel suggereren dat $ß_3$-adrenoreceptoren betrokken zijn bij de remming van de NANC-neurotransmissie.

De betrokkenheid van de cholinerge tonus bij het optreden van nachtelijk astma wordt ondersteund door een studie waarbij intraveneuze toediening van atropine, resulterend in een adekwate cholinerge blokkade, de nachtelijke luchtwegvernauwing bij volwassenen met astma aanmerkelijk verminderde. Een verhoogde perifere aktiviteit speelt hierbij waarschijnlijk een belangrijker rol dan een verhoogde centrale cholinerge aktiviteit. Dit kan worden gekonkludeerd uit tegenstrijdige resultaten die werden gevonden over de aanwezigheid van een verhoogde centrale cholinerge circadiane variatie bij volwassenen met astma, gemeten aan variaties in de hartslag en de 'sinus arrhythmia gap'. Daarnaast werd gevonden dat na hart-longtransplantatie, waarbij de centrale vagale innervatie van de luchtwegen verloren gaat, nog steeds een diurnaal ritme in luchtwegdiameter bestaat. Het is mogelijk dat de gevoeligheid van de cholinerge neurotransmissie voor inflammatoire en ander prikkels 's nachts verhoogd is tengevolge van een opgeheven remming van dit systeem door lagere spiegels van het

circulerend adrenaline. Een verhoogde lokale cholinerge aktiviteit kan worden bewerkstelligd door een verhoogde efferente stimulatie, bijvoorbeeld doordat inflammatoire mediatoren de negatieve feedback van de neurotransmissie via M_2-receptoren, en mogelijk andere remmende receptoren, antagoneren. Daarnaast kunnen inflammatoire en andere prikkels via afferente sensoire stimulatie een verhoogde cholinerge aktiviteit bewerkstelligen.

De NANC-tonus vertoont eveneens een circadiane variatie. In tegenstelling tot personen zonder nachtelijk astma, trad bij personen met nachtelijk astma geen circadiane variatie in bronchodilatatie op na stimulatie met capsaicine, hetgeen suggereert dat de NANC-neurotransmissie bij deze personen verstoord is. Het is bekend dat inflammatoire mediatoren de NANC-neurotransmissie op velerlei wijzen kunnen beïnvloeden. De bijdrage van dit systeem aan het optreden van nachtelijk astma dient verder te worden onderzocht, bijv. met behulp van specifieke remmers van de NANC-neurotransmissie.

Voor een rol van de nachtelijke daling van het circulerend cortisol in het optreden van nachtelijk astma is tot nu toe weinig bewijs voorhanden. Een direkt effekt van endogeen cortisol op de luchtwegdiameter is niet waarschijnlijk, zoals blijkt uit een studie waarin de nachtelijke daling van de cortisolspiegel werd gecorrigeerd door fysiologische doses hydrocortison intraveneus toe te dienen, zonder dat dit de nachtelijke daling in luchtwegdiameter bij vijf van de zes deelnemers voorkwam. Het is echter bekend dat corticosteroïeden de inflammatoire respons in de submucosa van de luchtwegen onderdrukken en het kan niet worden uitgesloten dat de midnachtelijke daling van het circulerende cortisol resulteert in een versterking van de lokale inflammatoire respons in de luchtwegen, hetgeen indirekt kan leiden tot verslechtering van de luchtwegdiameter 's nachts. Meer onderzoek is nodig om de invloed van circadiane schommelingen in cortisolspiegels op inflammatoire aktiviteit in de submucosa van de luchtwegen op te helderen.

In de klinische praktijk kan nachtelijk astma met de huidige kennis worden beschouwd als een uitingsvorm van ernstiger astma. De behandelingsstrategie zal derhalve grotendeels overeenkomen met de therapie van astma in het algemeen. De therapie is primair gericht op eliminatie van provocerende faktoren, bijvoorbeeld reductie van allergeenexpositie bij atopische personen en een adekwate behandeling van een gediagnosticeerde gastro-oesofageale reflux. Indien deze maatregelen niet afdoende

zijn, is het nuttig om reductie van inflammatoire aktiviteit in de luchtwegen te bewerkstelligen door het voorschrijven van anti-inflammatoire medicijnen. Indien dit ook niet voldoende helpt, is de volgende stap het toevoegen van luchtwegverwijders die lang genoeg werken om de slaapperiode te overbruggen.

2. Inflammatoire processen en AMP luchtweghyperreaktiviteit

Uit de studies over luchtweghyperreaktiviteit in dit proefschrift blijkt dat het meten van de AMP-geïnduceerde luchtweghyperreaktiviteit, naast de metacholine-geïnduceerde luchtweghyperreaktiviteit, aanvullende informatie geeft over de pathogenetische mechanismen die ten grondslag liggen aan de luchtwegvernauwing bij nachtelijk astma en over de ziekteaktiviteit bij COPD en astma. AMP inhalatieprovokatie-testen lijken derhalve een waardevolle en veelbelovende parameter in het laboratoriumonderzoek voor het *in vivo* vervolgen van inflammatoire aktiviteit in de luchtwegen en het evalueren van het effekt van anti-inflammatoire medikamenteuze therapie in astma en COPD. AMP inhalatieprovokatie-testen hebben gedurende de laatste jaren een plaats verworven in onze lopende onderzoeksprojekten. In de nabije toekomst zullen we dus in staat zijn om de meerwaarde van deze parameter op een grotere schaal te evalueren. Een belangrijke beperking in de toepassing van AMP inhalatie-provokatietesten blijft echter de interpretatie van de resultaten, omdat niet zeker is welke mechanismen precies betrokken zijn bij de AMP-geïnduceerde luchtwegvernauwing. Het laatste decennium zijn een aantal studies gepubliceerd die een rol voor mestcel-uitstorting ondersteunen. Het is desondanks belangrijk om te realiseren dat andere mechanismen, waaronder neurale reflexmechanismen, een even zo grote rol kunnen spelen terwijl dit tot nu toe niet onderkend is, omdat de mogelijkheden om deze mechanismen goed te onderzoeken gelimiteerd zijn. Meer onderzoek is nodig om de mechanismen die betrokken zijn bij de AMP-geïnduceerde luchtwegvernauwing verder op te helderen.

Konklusies

De belangrijkste bevindingen van de onderzoeken die in dit proefschrift werden beschreven zijn:

1. Uit literatuurstudie blijkt dat er correlaties bestaan tussen metacholine- of histamine-geïnduceerde luchtweghyperreaktiviteit en verschillende inflammatoire celparameters, maar deze correlaties zijn niet eenduidig. Op dit moment bestaat geen geschikte stimulus die kan worden gebruikt als een parameter van specifieke

ontstekingsprocessen in de luchtwegen.

Inflammatoire processen en nachtelijk astma

2. Het optreden van een toegenomen nachtelijke luchtwegobstruktie in atopische personen met astma is significant gecorreleerd met een toegenomen indirekte en direkte luchtweghyperreaktiviteit overdag, alsmede met de (16.00 - 04.00 uur) verandering in de PC_{20} AMP-waarde. De PC_{20} AMP-waarde daalde 's nachts meer dan de PC_{20} metacholinewaarde bij astmapatiënten met een toegenomen nachtelijke luchtwegvernauwing. Deze bevindingen suggereren dat inflammatoire mechanismen een rol spelen bij het optreden van nachtelijk astma.
3. Propranololinhalatie om 8.00 uur 's ochtends bij personen met atopisch astma gaat de normale diurnale stijging van de FEV_1 tegen. Dit suggereert indirekt een rol voor circulerend adrenaline in de diurnale modulatie van de luchtwegdiameter.
4. Een toegenomen nachtelijke luchtwegvernauwing bij personen met atopisch astma blijkt niet geassocieerd met dag-nacht veranderingen in celaantal en -aktiviteit in BAL-vloeistof en in perifeer bloed. Overdag kan de atopische astmagroep met toegenomen nachtelijke luchtwegvernauwing worden onderscheiden van die zonder toegenomen nachtelijke luchtwegvernauwing door een verhoogde aktiviteit van een aantal specifieke ontstekingscellen in BAL-vloeistof en perifeer bloed.
5. In slijmvliesbiopten van personen met atopisch astma wordt een toename van $CD4^+$ cellen 's nachts gezien. Verder onderzoek is geboden naar verschillen tussen astmatische personen met en zonder nachtelijke luchtwegvernauwing.
6. Het optreden van nachtelijk astma is geassocieerd met een verhoogde ontstekingsaktiviteit in de luchtwegen overdag en kan derhalve worden beschouwd als een uitingsvorm van ernstiger astma.

Inflammatoire processen en AMP-luchtweghyperreaktiviteit

7. Inhalatieprovokatietesten met AMP veroorzaken luchtwegvernauwing in de meeste patiënten met COPD, maar de respons is minder sterk ten opzichte van patiënten met astma met een vergelijkbare PC_{20} metacholinewaarde. De bevindingen suggereren dat inflammatoire mechanismen een rol spelen bij het ontstaan van luchtwegvernauwing bij COPD. Daarnaast lijken ontstekingsprocessen veroorzaakt door roken de mate van AMP luchtweg-hyperreaktiviteit bij COPD te bepalen. Daarentegen blijken rokers met astma juist minder gevoelig voor AMP provokatie dan niet-rokers met astma. Het bepalen van de PC_{20} AMP lijkt een gevoeliger maat van ziekte-aktiviteit dan de PC_{20} metacholine bij personen met atopisch astma.

Dankwoord

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Zwolle, augustus 1994
Ytske Oosterhoff

Curriculum vitae van de schrijfster van dit proefschrift

1972-1978	Atheneum B, Rijksscholengemeenschap Drachten.
1978-1985	Geneeskunde, Rijks Universiteit Groningen.
1985-1986	Keuringsarts, Bloedbank Groningen-Drenthe.
1986-1987	Arts-assistente chirurgie/interne geneeskunde/cardiologie/ kindergeneeskunde Wilhelmina Ziekenhuis Assen.
1987-1989	Wetenschappelijk onderzoek: 'Aktivatie van bronchoalveolaire fagocyten tijdens uitstoting van longtransplantaten bij de rat'. Experimentele thoraxchirurgie AZG. Gesubsidieerd door de Jan Kornelis de Cock-Stichting.
1989-1990	Wetenschappelijk onderzoek: 'Verstoring van de synthese van bindweefselkomponenten in de pathogenese van humaan longemfyseem'. Longziekten AZG.
1990-1993	Wetenschappelijk onderzoek: 'Inflammatie bij nachtelijke bronchusobstructie en hyperreactiviteit'. Longziekten AZG. Gesubsidieerd door het Nederlands Astma Fonds.
1991	Postgraduate course: 'Immunology of asthma'. University of London. Gesubsidieerd door Glaxo.
1993-1995	Basisopleiding interne geneeskunde. Sophia Ziekenhuis Zwolle.
1995-	Opleiding tot longarts. Longziekten AZG.