Sputum Eicosanoid Profiling in Exacerbations of Chronic Obstructive Pulmonary Disease

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Key Words

Biomarker · Inflammation · Oxidative stress · Prostaglandin E₂ · Repeatability · Treatment

Abstract

Background: Eicosanoids are small lipid molecules with diverse biological functions in the airways. **Objectives:** The aim of this study was to investigate changes in leukotriene B₄ (LTB₄), 8-isoprostane, prostaglandin E₂ (PGE₂) and cysteinyl-leukotriene (cys-LT) levels in the sputum of patients with chronic obstructive pulmonary disease (COPD) at the onset of a severe exacerbation and during the course of recovery. **Methods:** Thirty-seven ex-smoker COPD patients suffering an episode of acute exacerbation were enrolled. Samples were taken (i) on hospital admission and (ii) after regular treatment. Twenty-five stable ex-smoker COPD patients served as controls. Eicosanoids were determined by enzyme immunoassay. **Results:** Sputum PGE₂ [39.8 (13.3–103.3) vs. 5.05 (2.3–12.1) pg/ml, $p < 0.001$], 8-isoprostane [89.5 (36.9–184.7) vs. 29.7 (13.8–68.8) pg/ml, $p < 0.01$] and LTB₄ [587.7 (252.9–774.8) vs. 276.1 (105.4–594.7) pg/ml, $p < 0.05$] levels were increased in patients with exacerbation compared to stable subjects. After treatment only PGE₂ levels decreased significantly [at discharge: 19.6 (4.6–52.5) pg/ml, $p < 0.01$], the levels of other eicosanoids remained elevated ($p = \text{NS}$). Sputum cys-LT levels were similar in stable patients and in those with exacerbation and treatment did not influence

cys-LTs either. There was a significant correlation between PGE₂ and sputum neutrophil and lymphocyte cell counts in patients with exacerbation. **Conclusions:** Our results suggest that 8-isoprostane, LTB₄ and PGE₂ but not cys-LTs may be involved in exacerbation-associated inflammatory processes in the airways of patients with COPD. Validation of PGE₂ for use as a biomarker of recovery from an exacerbation requires further studies.

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Introduction

Acute exacerbations of chronic obstructive pulmonary disease (COPD) are associated with increased inflammation and mediator release into the airways [1]. Among mediators involved in the development of exacerbations, arachidonic acid metabolites such as leukotrienes (LTs) and prostaglandins (PGs) may play an important role [2].

Several lines of evidence indicate that LTB₄ acts as a potent neutrophil chemoattractant during exacerbations [3, 4]. In asthma it has been extensively documented that cysteinyl-LTs (cys-LTs) including LTC₄, LTD₄ and LTE₄ induce bronchoconstriction, increase mucus production and edema and promote airway remodeling [5] while PGE₂ is an airway smooth muscle relaxant and is likely to have bronchoprotective and anti-inflammatory actions [6]. In COPD the role of these mediators is less clear.

Fig. 1. Flow chart showing the study profile in patients with exacerbations of COPD.

Montuschi et al. [7] reported an increased PGE₂ concentration in exhaled breath condensate (EBC) of stable COPD patients and argued that, like in asthma, this might represent a protective mechanism for counteracting airway inflammation. However, PGE₂ has also been implicated as an important mediator of cigarette smoke-induced neutrophil infiltration of the airways, indicating that PGE₂ may have diverse and even opposing effects in respiratory diseases [8]. Moreover, an increase in cys-LTs in EBC [9] and a prominent cys-LT1 receptor expression [10] have also been recently demonstrated during the course of an exacerbation.

Isoprostanes represent a unique group of arachidonic acid derivatives, since they are produced nonenzymatically from arachidonic acid during the peroxidation of membrane lipids, and in addition to having relevant biological activities, may also be potentially useful biomarkers of oxidative stress [11]. In COPD exacerbations raised 8-isoprostane levels in EBC indicate augmented oxidative stress [9, 12, 13].

Until recently, most studies had used EBC as a type of respiratory sample to profile eicosanoids in COPD exacerbations [9, 12, 13]. However, measurement of putative mediators in EBC usually have poor reproducibility [14, 15]. Moreover, biomarkers may be affected by the sam-

pling procedure itself, as acknowledged in the European Respiratory Society Task Force Report [16].

Assessment of eicosanoids in the sputum supernatant is a more reliable approach to study the underlying pathophysiology and to identify pulmonary biomarkers of potential clinical utility in the management of the disease [17]. Nevertheless, major eicosanoid products in the sputum have not yet been simultaneously investigated in COPD exacerbations and in convalescence. Therefore, in this study, levels of LTB₄, cys-LTs, PGE₂ and 8-isoprostane in sputum were assessed in patients with COPD exacerbation at onset and after treatment.

Materials and Methods

Study Subjects

COPD patients with an acute exacerbation of the disease were recruited for the study between January 2012 and December 2012. Inclusion and exclusion criteria are summarized in figure 1. COPD exacerbation was defined as increased dyspnea, cough or sputum expectoration that led the subject to seek medical attention, as specified in international guidelines [18].

For comparison 25 clinically stable, ex-smoker COPD patients were additionally enrolled in the study (table 1). All patients were >40 years of age and had a smoking history of >10 pack-years and documented airway obstruction with forced expiratory volume in

Table 1. Demographic and clinical characteristics of study subjects

	Stable COPD	COPD exacerbation	
		on admission	after treatment
Subjects, n	25	37	
Sex, male/female, n	15/10	22/15	
Age, years	62.1±1.6	64.6±2.2	
Smoking, pack-years	48.8±4.8	45.8±4.1	
GOLD stages, n			
I	5 (20%)	5 (13%)	
II	12 (48%)	14 (38%)	
III	8 (32%)	11 (30%)	
IV	–	7 (19%)	
Pulmonary function			
FVC, liters	3.55±0.21	1.93±0.13 [§]	2.23±0.17 [#]
FVC, % predicted	84.7±3.0	65.4±3.4 [§]	76.5±4.0
FEV ₁ , liters	2.91±0.11	0.92±0.08 [§]	1.14±0.10 [§]
FEV ₁ , % predicted	63.4±4.5	40.2±2.7 [§]	49.6±3.2 [§]
FEV ₁ /FVC, %	50.8±2.1	48.2±3.3	53.7±2.5*
Blood gases			
PaCO ₂ , kPa	5.14±0.11	5.54±0.13	5.80±0.14
PaO ₂ , kPa	8.40±0.21	6.81±0.20 [§]	7.16±0.15*
FENO, ppb	8.6 (5.6–9.9)	13.1 (8.4–26.5)	10.7 (6.9–17.3)*

Data are presented as mean ± SEM unless stated otherwise. FVC = Forced vital capacity; FENO = fractional exhaled nitric oxide (median, interquartile ranges); ppb = parts per billion; PaCO₂ = arterial carbon dioxide tension; PaO₂ = arterial oxygen tension.

* p < 0.05, # p < 0.01 or § p < 0.001 vs. admission, § p < 0.001 vs. stable COPD.

1 s (FEV₁) <80% of predicted and postbronchodilator FEV₁/forced vital capacity <0.7. The research protocol was approved by the local Ethics Committee and all subjects gave written informed consent to participation in the study.

Study Design

In patients with exacerbation, spontaneously expectorated sputum was collected and levels of fractional exhaled nitric oxide, blood gases and lung function parameters were measured on hospital admission and on the day of discharge. In stable COPD patients induced sputum samples were collected during routine clinical visits. Lung function, blood gas parameters and fractional exhaled nitric oxide were determined, as previously described [19].

Sputum Induction and Processing

In stable COPD sputum was induced by the inhalation of a hypertonic saline solution (4% sodium chloride) delivered by an ultrasonic nebulizer (Ultra-Neb 2000, DeVilbiss Healthcare Ltd., Tipton, UK) with an output set at 1 ml/min.

Spontaneous and induced sputum samples were processed similarly within 120 min from collection, as previously described [20]. At least 400 inflammatory cells were counted for each cytospin slide. The inflammatory cells in sputum were

shown as a percentage of total nonsquamous cells (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000358099).

Enzyme Immunoassays

LTB₄, 8-isoprostane, cys-LTs and a stable derivative of PGE₂ were determined in sputum supernatants by enzyme immunoassays (EIA, Cayman Chemical, Ann Arbor, Mich., USA) according to the manufacturer's protocol, with the modification that dithiothreitol of the same concentration as in the sputum supernatant (0.04%) was added to the standards in agreement with other studies [21]. The concentrations of eicosanoids were corrected for dilutions. The detection limits for LTB₄, 8-isoprostane, cys-LTs and PGE₂ in dithiothreitol-treated sputum supernatants were 18, 4, 28 and 2 pg/ml, respectively.

The repeatability of EIA measurements in the sputum was determined in a pilot study. Sputum samples collected from a subset of COPD patients (n = 6) were divided into two aliquots, which were analyzed separately (online suppl. table 2).

Comparison of Eicosanoid Levels between Induced and Spontaneous Sputum

To compare eicosanoid levels in induced and spontaneous sputum, a subgroup of stable COPD patients (n = 10) capable of expectorating sputum spontaneously was selected. From these subjects both induced and spontaneous sputum samples were collected.

Statistical Analysis

Data are presented as mean ± SEM or median with interquartile ranges when appropriate. Eicosanoid levels between stable and exacerbation patients were analyzed by the Mann-Whitney test. Paired Student's t test (parametric data) and the Wilcoxon signed rank test (nonparametric data) were used to compare variables measured on hospital admission and discharge. Correlation coefficients were calculated by Spearman's method. The repeatability of the EIA measurements was estimated by the coefficient of variation and the limits of agreement (Bland-Altman test). Power calculation was performed with α = 0.05 and 0.87 effect size. Calculations were performed by GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, Calif., USA) and G*Power 3.1.1 (G*Power Software Inc., Kiel, Germany) software packages. A p value <0.05 was considered significant.

Results

Fifty-two COPD patients with exacerbation were screened, 45 fulfilled inclusion criteria and agreed to participate (fig. 1). During hospital treatment 8 patients were withdrawn. Demographic and clinical data of the 37 patients who completed the study are presented in table 1.

Clinical Variables during Treatment of Exacerbations

Exacerbations were treated with systemic glucocorticoids and bronchodilators (anticholinergics and/or β₂-agonists) in all cases. Antibiotics were given to 25 COPD

Table 2. Lipid mediator concentrations in induced and spontaneous sputum samples

Mediators, pg/ml	Sputum			CV, %	Limits of agreement ^a
	spontaneous	induced	p value		
8-Isoprostane	183.0 (4.1–1,233)	195.7 (3.4–1,299)	0.937	18.9	–179.1 and 127.2
cys-LTs	446.5 (50.9–1,563)	399.2 (151.3–1,563)	0.902	22.0	–204.9 and 281.7
PGE ₂	28.7 (6.6–80.6)	22.4 (2.2–81.4)	0.605	21.8	–13.2 and 22.1
LTB ₄	973.5 (418.9–1,654)	907.1 (346.2–1,500)	0.798	14.8	–245.0 and 383.1

Data are presented as median (interquartile ranges) unless stated otherwise. CV = Coefficient of variation.

^a Determined by the Bland-Altman test.

patients. The mean length of hospitalization was 12.0 ± 2.9 days.

Lung function variables and PaO₂ increased (table 1), while sputum total cell counts and the number of neutrophils decreased during the course of treatment (online suppl. table 1). By contrast, inflammatory cell type ratios were not changed.

Comparison of Eicosanoid Levels between Induced and Spontaneous Sputum

Eicosanoid levels obtained in spontaneous and induced sputum samples are given in table 2. Mediator levels in the two types of samples were similar.

8-Isoprostane

Apart from 1 patient in the stable COPD group, 8-isoprostane levels were above the detection limit in all subjects. Concentrations were significantly higher in patients with exacerbations compared to stable subjects [89.5 (36.9–184.7) vs. 29.7 (13.8–68.8) pg/ml, $p < 0.01$; fig. 2a], and remained elevated after treatment of exacerbation [84.1 (34.1–185.3) pg/ml].

Cysteinyl-Leukotrienes

Cys-LTs were detectable in all samples. Levels were slightly higher in patients with exacerbations, however, differences did not reach statistical significance [211.9 (128.6–827.1) vs. 469.9 (169.2–906.8) pg/ml, $p = \text{NS}$; fig. 2b]. Treatment had no effect on cys-LT concentrations [324.2 (114.3–698.1) pg/ml, $p = \text{NS}$].

Prostaglandin E₂

In the stable COPD group, PGE₂ levels were detectable only in 13 patients. Among patients with exacerbation PGE₂ was detectable in all cases. PGE₂ concentrations were significantly higher in patients with exacerbation than in stable subjects [39.8 (13.3–103.3) vs. 5.05 (2.3–

12.1) pg/ml, $p < 0.001$; fig. 2c]. Levels significantly decreased along with treatment [19.6 (4.6–52.5) pg/ml, $p < 0.01$].

Leukotriene B₄

Among stable COPD patients LTB₄ was detectable in all samples. Levels were increased in patients with exacerbation compared to stable subjects [587.7 (252.9–774.8) vs. 276.1 (105.4–594.7) pg/ml, $p < 0.05$; fig. 2d]. Treatment had no effect on LTB₄ concentrations [661.5 (182.6–758.1) pg/ml, $p = \text{NS}$] by the time of discharge. The power of the study to detect a standardized difference in 8-isoprostane, cys-LT, PGE₂ and LTB₄ between stable patients and those with exacerbation was 89, 90, 83 and 89%, respectively.

Correlations

Major correlation data are summarized in table 3. Briefly, in stable patients significant correlations were observed between the number of neutrophils and 8-isoprostane or LTB₄ concentrations in the sputum. Moreover, lymphocyte cell counts showed an association with 8-isoprostane and cys-LT levels.

In patients with exacerbations significant correlations were observed between sputum PGE₂ levels and neutrophil or lymphocyte cell counts. Similarly, LTB₄ and cys-LT levels presented an association with the number of neutrophils. Other sputum cell types, spirometric and other clinical variables showed no correlations with lipid mediator levels (data not shown).

Discussion

In this study we investigated the sputum eicosanoid profile of COPD patients hospitalized with an acute exacerbation. The most striking finding of the study was that

Fig. 2. Sputum 8-isoprostane (a), cys-LTs (b), PGE₂ (c) and LTB₄ (d) concentrations in stable COPD patients (Stable) and in COPD patients hospitalized at the time of an acute exacerbation (Ex) and after hospital treatment (Treat). In 3 patients with exacerbation

LTB₄ and PGE₂ levels were higher than the detection range of the kit. All patients were ex-smokers. Horizontal bars represent median values. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. stable COPD patients, # $p < 0.01$ vs. exacerbation.

Table 3. Correlations between sputum lipid mediator concentrations and clinical variables or sputum inflammatory cells in stable COPD patients and in those with exacerbations

Variables	8-Isoprostane		cys-LTs		PGE ₂		LTB ₄	
	r value	p value	r value	p value	r value	p value	r value	p value
Stable COPD								
FEV ₁ , % predicted	-0.18	0.39	-0.13	0.52	-0.45	0.10	0.20	0.35
Neutrophils	0.61	0.004 ^a	0.39	0.07	0.18	0.53	0.52	0.013 ^a
Lymphocytes	0.64	0.002 ^a	0.60	0.003 ^a	0.27	0.35	0.38	0.084
COPD exacerbations								
FEV ₁ , % predicted	0.07	0.75	-0.37	0.07	0.03	0.91	-0.37	0.11
Neutrophils	-0.05	0.82	0.54	0.003 ^a	0.61	0.001 ^a	0.45	0.024 ^a
Lymphocytes	0.07	0.72	0.55	0.002 ^a	0.69	<0.001 ^a	0.24	0.25

^a Significant correlations.

PGE₂ levels sharply increased at the onset of exacerbations compared to those in stable patients. Treatment of the condition resulted in a decrease in PGE₂ concentrations. LTB₄ and 8-isoprostane levels were also increased in exacerbations; however, levels remained elevated by the time of hospital discharge after treatment.

In contrast to PGE₂, LTB₄ or 8-isoprostane, sputum cys-LT concentrations were not elevated in COPD patients with exacerbations, indicating that cys-LTs may not significantly contribute to the development of airflow obstruction during exacerbations. Importantly, our study was sufficiently powered to detect a clinically meaningful change in cys-LT between a stable state and an exacerbation. Different mediator expressions could be present between eosinophilic and noneosinophilic airway inflammation [22]. Nonetheless, in our study, subgroup analysis based on the type of inflammation [eosinophilic (>2%) vs. noneosinophilic (<2%)] revealed no change in cys-LTs either (data not shown).

Our finding that PGE₂ concentrations in the sputum were increased in COPD exacerbations deserves comments. On the one hand, it is possible that this is merely a secondary protective regulatory response, as has been suggested in asthma [23] or COPD [7]. However, it is also conceivable that PGE₂ is involved in neutrophilic recruitment into the airways, since there is evidence that PGE₂ can enhance the adhesion of neutrophils to bronchial epithelial cells via a cyclooxygenase-2-dependent mechanism in COPD patients [8]. In line with this theory, PGE₂ concentrations correlated with the number of neutrophils present in sputum samples at the time of exacerbation in our study. Interestingly, the number of lymphocytes showed an association with PGE₂ levels as well. Finally, it is also possible that PGE₂ influences airway inflammation and parenchymal destruction through the regulation of matrix metalloproteinases, since some studies suggest a relationship between the production of PGE₂ and matrix metalloproteinase-2 in the airways of COPD patients [24].

Although PGE₂ concentrations decreased along with treatment of our patients, at discharge they were still markedly higher compared with those of stable COPD patients. It is possible that this was simply due to the high degree of neutrophilic airway inflammation present at this time point in our patients. Indeed, sputum neutrophil counts were elevated in patients at discharge compared to stable subjects and neutrophils represent a major source of PGE₂ in the lung, as a result of induction by inflammatory cytokines such as TNF- α and IL-1 β , which activate NF- κ B, the key regulator of cyclooxygenase-2

[25]. These inflammatory mediators might still be up-regulated by the time of discharge and promote PGE₂ synthesis in other cell types, for example, in fibroblasts as well [26]. Moreover, it has been reported that *Moraxella catarrhalis* and *Haemophilus influenzae*, which are major causes of infectious COPD exacerbations, can induce PGE₂ release in pulmonary epithelial cells via a cyclooxygenase-2-dependent mechanism [27, 28]. It is possible that some of our patients were infected with these respiratory pathogens, which could persist even by the time of discharge. Nevertheless, sputum culture results were not specifically investigated in this study.

It is of note that in vivo PGE₂ is rapidly converted to an inactive metabolite (13,14-dihydro-15-keto PGE₂) by the PG 15-dehydrogenase pathway. Therefore, the determination of in vivo PGE₂ biosynthesis is often best accomplished by the measurement of PGE₂ metabolites. Thus, the EIA assay that was employed in our study first converts all major PGE₂ metabolites into a single stable derivative which allows reliable estimate of in vivo PGE₂ levels.

Our results about cys-LTs and PGE₂ contrast with previous work assessing these eicosanoids in EBC [9]. We speculate that either the different nature of the specimens used (sputum vs. EBC) or the differences in the study cohort may explain the discrepancy between the results. In the study by Antczak et al. [9] only infectious exacerbations were investigated, while in our study positive sputum culture was not an inclusion criterion. The repeatability of sputum biomarker measurements such as 8-isoprostane and LTB₄ in sputum is better than those reported by others for the same biomarkers in EBC [14].

Our finding that COPD patients with exacerbations have higher sputum LTB₄ concentrations than stable subjects confirms the results of some previous studies [3, 4]. Similarly, the significant correlation between sputum neutrophils and LTB₄ levels is consistent with the general view that LTB₄ is involved in neutrophil recruitment in COPD [29, 30] or asthma [31]. Interestingly, treatment had not resulted in a reduction of LTB₄ concentrations. Nonetheless, delayed resolution of inflammatory response during recovery from exacerbation has been observed by other investigators as well [32]. Moreover, changes in sputum lipid mediator levels are not always directly related to anti-inflammatory treatment [33].

Earlier studies showed higher 8-isoprostane levels in the EBC and/or the sputum of stable COPD patients compared to healthy controls [34, 35]. Here, we found that 8-isoprostane levels were further elevated in COPD exacerbations. To the best of our knowledge, this has previ-

ously been demonstrated only in EBC [9, 12, 13]. Our findings are in agreement with those of other trials showing increased oxidative stress in COPD patients with exacerbations using other markers such as exhaled hydrogen peroxide [36] or α_1 -antitrypsin [37]. However, sputum may be more suitable for assessing oxidative stress in respiratory diseases since the levels of potential markers including 8-isoprostane are 5- to 10-fold higher in the sputum than in the EBC [17]. Our data also indicate that a successful hospital treatment resulting in clinical and functional recovery of the patient does not abolish the increased oxidative stress observed in COPD exacerbations by the time of the patient's discharge from the hospital. Further studies with a longer follow-up period are needed to specifically investigate this issue.

In our study lung function parameters were not related to sputum lipid mediator levels. In other series investigating patients with asthma [38] or COPD exacerbations [9, 12] no such correlations were found either. Similarly, the association between lung function and sputum cytokine concentrations is often inconsistent [39].

We are aware that sputum induction, as opposed to spontaneous sputum collection, is the method of choice in patients with mild to moderate COPD [40]. However, as a large percentage (~50%) of our recruits had severe or very severe COPD, spontaneous sputum collection, rather than sputum induction was chosen in patients with exacerbation because of safety concerns. Since the induc-

tion by itself had no effect on eicosanoid readings, direct comparison of the induced and spontaneous sputum specimens was feasible.

Several lines of evidence indicate that cigarette smoking is a confounding factor in eicosanoid measurements [8, 34, 41]. Since our recruitment strategy called for only ex-smokers, smoking status had no influence on our results.

In conclusion, we found elevated PGE₂ concentrations in the sputum in relation with increased airway inflammation in patients with COPD exacerbations. PGE₂ decreased along with clinical recovery. During exacerbations sputum 8-isoprostane levels were also elevated indicating augmented oxidative stress. Finally, while increased LTB₄ could contribute to enhanced neutrophilic influx during exacerbations, no evidence for a major role of cys-LTs in exacerbations was found. The utility of PGE₂ measurements in the sputum should be investigated further to clarify its possible role as a marker of recovery from a COPD exacerbation.

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