

Exhaled 8-isoprostane in sarcoidosis: relation to superoxide anion production by bronchoalveolar lavage cells

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Abstract

Objective This study was designed to examine the mutual relationship between 8-isoprostane in exhaled breath condensate (EBC) and superoxide anion generation by bronchoalveolar lavage fluid (BALF) cells in patients with sarcoidosis.

Design About 29 patients with sarcoidosis, 34 healthy never smokers (control group for EBC) and 15 healthy never smokers (control group for BAL) were examined. EBC was collected directly before bronchoscopy. 8-Isoprostane was measured by ELISA, and superoxide anion by colorimetry.

Results Exhaled breath condensate 8-isoprostane is increased in sarcoidosis (median, 25–75 percentile): 2.50; 2.50–3.90 versus 6.20; 2.50–16.95 pg/ml, $p \leq 0.05$). Spontaneous superoxide anion release from BALF cells was significantly elevated only in patients with a high percentage of lymphocytes in BALF (6.42 ± 1.24 vs. 23.52 ± 4.30 nmol/ 10^6 cells, $p \leq 0.01$). There were no correlations between 8-isoprostane and spontaneous or stimulated superoxide anion release.

Conclusions We confirmed higher concentrations of EBC 8-isoprostane in sarcoidosis and higher spontaneous release of superoxide anion from BALF cells in patients with sarcoidosis. The increase of EBC 8-isoprostane is not directly related to superoxide anion released from BALF cells.

Keywords Bronchoalveolar lavage · Exhaled breath condensate · Oxidative stress · Sarcoidosis · Superoxide anion

Introduction

Oxidative stress has been implicated in the pathophysiology of many lung diseases. An example is idiopathic pulmonary fibrosis (IPF), a disease characterized by fatal prognosis due to massive lung fibrosis and unresponsiveness to treatment [1]. Increased ex-vivo production of superoxide anion or hydrogen peroxide by cells from bronchoalveolar lavage (BAL) have also been reported in sarcoidosis [2, 3], but secondary markers of oxidative stress, such as oxidized proteins, or oxidized methionine residues in BAL fluid (BALF) are not increased in sarcoidosis, contrary to IPF [4–6].

Exhaled breath condensate is a relatively new, promising and non-invasive method that enables collecting biological material from lower respiratory tract in order to measure various biomarkers [7]. 8-Isoprostane is a marker of oxidative stress. It is a prostaglandin- $F_{2\alpha}$ isomer, produced in vivo by free radical-catalyzed peroxidation of arachidonic acid [7–9]. Elevated concentrations of 8-isoprostane in BALF [10] and exhaled breath condensate (EBC) [11, 12] in patients with sarcoidosis have been reported. However, the clinical value of this finding is still unknown. There is some hope that this biomarker could be

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helpful in selecting patients with the worst prognosis. Reactive oxygen species (ROS) activate metalloproteinases and inactivate their inhibitors [13, 14], or stimulate cells to release TGF- β and other pro-fibrogenic cytokines [15]. Also, alveolar epithelial cells apoptosis can be induced by ROS [16]. All these mechanisms may be involved in the pathogenesis of chronic and progressive lung sarcoidosis.

We have previously reported increased 8-isoprostane in exhaled breath condensate in patients with sarcoidosis [12]. Further, we found that spontaneous, rather than PMA-stimulated, superoxide anion production by BAL cells is increased in sarcoidosis [17]. The present study was planned to evaluate the correlation between both spontaneous and PMA stimulated superoxide anion generation and EBC 8-isoprostane.

Materials and methods

Study and control groups

Twenty-nine patients with newly diagnosed, histo-pathologically confirmed sarcoidosis were included. All were non-smokers, non-atopic and had never been treated with steroids or immunosuppressive drugs. There were no other co-morbidities. Respiratory infection in the last 4 weeks was an exclusion criterion. Based on the radiological classification, there were 9 patients with stage I, 14 with stage II, and 6 with stage III. The control group for EBC 8-isoprostane consisted of 34 healthy never-smokers, members of the hospital staff and medical students. The control group for superoxide release from BALF cells consisted of 15 healthy non-smokers who had undergone bronchoscopy due to unspecific clinical/radiological signs, finally proved not to be related to any lung pathology. Characteristics of the study and control groups are presented in Table 1.

Bronchoscopy was performed with a flexible bronchoscope (Pentax[®], Japan) according to British Thoracic

Society Guidelines [18]. Patients optionally received midanium and atropine before the examination, 2% lidocaine was used as a topical anaesthetic.

Bronchoalveolar lavage fluid (BALF) was collected from medial lobe or lingula by instillation and subsequent withdrawal of 4 \times 50 ml of 0.9% NaCl. The fluid recovery was 57 \pm 2%. The crude BALF was filtered through gauze, centrifuged, and the pellet was suspended in a phosphate buffer. The total cell count (TCC) was presented as $n \times 10^6$. Cytospin slides were prepared and stained by May-Grünwald-Giemsa stain. Numbers of macrophages, lymphocytes, neutrophils and eosinophils were calculated under a light microscope and presented as percent of TCC. Moreover, total cells and particular cell types were presented as number of cells ($n \times 10^4$) per ml of recovered fluid.

Superoxide anion (O_2^-) production by BALF cells was measured colorimetrically, as previously described [19]. The method is based on the reduction of cytochrome C by superoxide anion. Superoxide dismutase was used to inhibit the reaction. Spontaneous and excess release after stimulation with PMA (1 ng/ml, for 20 min) were measured. Values were expressed as nmol $O_2^-/10^6$ cells. Due to the fact that lymphocytes could be neglected as a source of oxidants, we presented these values as nmol $O_2^-/10^6$ cells potentially producing superoxide anion (macrophages, neutrophils, eosinophils). This value was calculated according to the following formula: $C = n \times 100/100 - y$, where n is the production of superoxide by all BALF cells, and y is a percentage of BALF lymphocytes.

Collection of exhaled breath condensate

The exhaled breath condensate (EBC) was collected using a condensing device (Ecoscreen, Jaeger, Germany). Patients were asked to breathe out spontaneously for 10 min through a mouthpiece equipped with a saliva trap. The respiratory rate ranged from 15 to 20 breaths/min. All

Table 1 Characteristics of the control and study groups

	Control EBC	Control BAL	Sarcoidosis all	Sarcoidosis stage I	Sarcoidosis stage II	Sarcoidosis stage III
Gender (females/males)	20/14	7/8	13/16	5/4	6/8	2/4
Age (years)	39.8 \pm 2.6	43.6 \pm 4.0	40.2 \pm 2.0	39.4 \pm 2.8	38.6 \pm 2.8	45.3 \pm 6.0
FEV ₁ % predicted	104.2 \pm 2.7	103.3 \pm 3.1	92.9 \pm 3.0	97.4 \pm 4.1	86.7 \pm 3.4	102.0 \pm 10.1
FVC % predicted	108.6 \pm 3.2	103.0 \pm 3.1	94.8 \pm 3.1	97.6 \pm 5.5	91.9 \pm 3.0	98.0 \pm 11.7
FEV ₁ /FVC %	81.2 \pm 1.1	84.4 \pm 1.6	84.9 \pm 1.5	89.0 \pm 2.2	81.5 \pm 1.9	87.2 \pm 3.3
DLCOc % predicted	–	–	83.2 \pm 3.6	91.1 \pm 5.1	79.7 \pm 4.6	73.4 \pm 13.8
BAL lymphocytes %	–	9.5 \pm 1.1	34.7 \pm 4.4	39.3 \pm 8.0	31.6 \pm 5.8	35.0 \pm 12.4

BAL bronchoalveolar lavage; DLCO diffusion capacity for carbon monoxide corrected for hemoglobin concentration; EBC exhaled breath condensate; FEV₁ forced expiratory volume in first second of expiration; FVC forced vital capacity

subjects wore a nose-clip and rinsed their mouths with distilled water just before and in the seventh minute of the condensing process in order to reduce nasal contamination. Samples were stored at -80°C for not longer than 4 weeks until measurements were taken. The collection of EBC was performed following available recommendations [20], always before the bronchoscopy.

8-Isoprostane concentrations in breath condensate were measured by a specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), as previously described [12]. The detection limit was 5 pg/ml. Levels of measured mediators below the detection limit were arbitrarily assumed to be half of the detection limit value. 8-Isoprostane was also measured in BAL, and results were used for estimation of correlations. BAL 8-isoprostane results for the control group are not available.

Statistical analysis

Data were expressed as mean \pm standard error of means (SEM). The Kolmogorow-Smirnoff test was used to assess normality. Median with 25th and 75th percentiles was provided for non-normally distributed data. Unpaired T-test (for normally distributed data) and Mann-Whitney test (for non-parametric data) were used to compare sarcoidosis with the controls. When more than two groups were compared, one-way ANOVA and Bonferroni post-test (for data with Gaussian distribution) or Kruskal-Wallis followed by Dunn's Multiple Comparison Test (for data without normal distribution) were used. The Spearman test was applied to assess correlations. A p value ≤ 0.05 was deemed statistically significant.

The study was approved by Ethical Committee at Medical University of Lodz (consent No. RNN/99/08/KE) and all patients signed informed consent.

The funding source had no influence on the study.

Results

Concentrations of 8-isoprostane in EBC were higher in sarcoidosis (median; 25–75 percentile: 2.50; 2.50–3.90 vs. 6.20; 2.50–16.95 pg/ml, $p < 0.05$). Although there were no significant differences between radiological stages, the significance was highest when stage III was compared with controls (26.35; 3.90–33.85 pg/ml, $p < 0.01$, Fig. 1) and between controls and patients with low lymphocyte percentage in BALF (15.10; 5.10–34.50 pg/ml, $p < 0.01$, Fig. 2a).

Superoxide anion release, both spontaneous (mean \pm SEM: 6.42 ± 1.24 vs. 17.08 ± 2.87 nmol/ 10^6 cells) and PMA-stimulated excess superoxide release (median; 25–75 percentile: 1.74; 0–6.01 vs. 5.90; 0–28.01 nmol/ 10^6 cells),

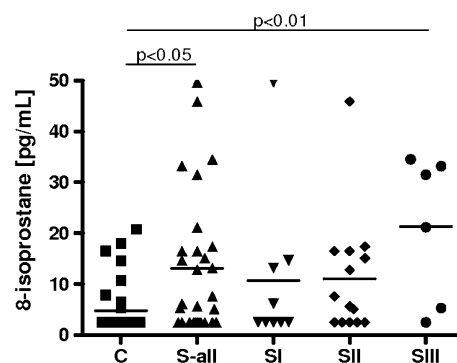


Fig. 1 Comparison of 8-isoprostane concentration in exhaled breath condensate (EBC) of controls (C), all sarcoidosis patients (S) and patients divided according to radiological stages (SI, SII and SIII)

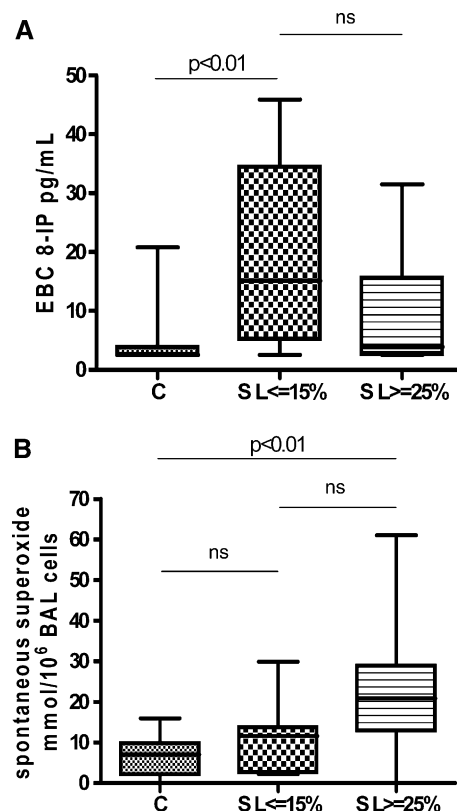


Fig. 2 EBC 8-isoprostane (a) and spontaneous superoxide anion release from BAL cells (b) depending on high and low lymphocyte percentage in BAL fluid versus controls. The bars are limited by 25th and 75th percentiles, lines within bars represent means, T-bars represent maxima. C controls; S sarcoidosis; L BAL lymphocytes ≤ 15 or $\geq 25\%$. Other abbreviations: BAL bronchoalveolar lavage; EBC exhaled breath condensate; ns not significant

in all sarcoidosis patients showed no significant difference compared to controls in this study. Neither in the case of spontaneous nor that of PMA-stimulated release were there any significant differences between patients with different radiological stages. Patients with high lymphocyte

percentage in BALF had significantly higher concentrations of spontaneous superoxide anion compared to controls (mean \pm SEM: 23.52 ± 4.30 nmol/ 10^6 cells, $p < 0.01$, Fig. 2b).

We did not find any correlations between EBC 8-isoprostane and spontaneous ($r = -0.06$; $p = 0.76$) or PMA-stimulated superoxide anion release ($r = -0.10$; $p = 0.61$). BAL 8-isoprostane was correlated with EBC results ($r = 0.64$, $p = 0.0004$), but was not correlated with spontaneous ($r = 0.10$, $p = 0.63$) or stimulated superoxide release ($r = 0.02$, $p = 0.92$).

Exhaled breath condensate 8-isoprostane concentrations were negatively correlated with the percentage of lymphocytes in BALF ($r = -0.40$; $p = 0.03$).

PMA-stimulated superoxide production was positively correlated with the number of neutrophils in BALF ($r = 0.48$; $p = 0.009$).

Bronchoalveolar lavage 8-isoprostane correlated negatively with FVC percent predicted ($r = -0.41$, $p = 0.049$) and FEV₁/FVC ratio ($r = -0.49$, $p = 0.02$).

Discussion

Although elevated concentrations of 8-isoprostanes in EBC [11, 12] and increased release of superoxide anion from alveolar cells after stimulation [2] in patients with sarcoidosis have already been reported, this is the first study on the mutual relationship between EBC 8-isoprostane and both PMA stimulated and spontaneous production of superoxide by BAL cells.

Despite the evident radiological and functional progression, several markers of activity are used in everyday practice, starting from various laboratory markers in serum or BAL, through increased Ga⁶⁷ uptake in scintigraphy scans, to changes in BALF cellular pattern. None of these markers is specific nor sensitive enough; in addition none adds much to the ability to determine future prognosis and indications to treatment. One of the best markers of activity, elevated percentage of lymphocytes in BALF (with high CD4/CD8 ratio), reflects only the intensity of lymphocytic alveolitis. Highly elevated content of lymphocytes is a typical feature of those with acute onset and evidently better prognosis [21, 22]. Other changes in BALF cellular pattern, however, may have some negative prognostic value. For instance, high content of neutrophils and eosinophils were reported in those with chronic course and increased risk of lung fibrosis [22, 23]. It may be important that these cells are very potent producers of reactive oxygen species (ROS) and other toxic substances, which may be released upon stimulation of NADPH-oxidase, or other ROS-generating intracellular sources. Our finding of higher spontaneous release of superoxide anion from BALF cells

of patients with active lymphocytic inflammation suggests that BALF macrophages are constantly stimulated *in vivo*, and so release superoxide anion spontaneously. Other authors also confirm such a possibility, showing spontaneous release of gamma-interferon from alveolar macrophages and lung T lymphocytes in sarcoidosis [24]. This type of ROS production seems to be related to the intensity of lymphocytic inflammation, and evidently is not a feature of a more severe disease. In clinical practice, however, the most problematic patients are those with low activity markers, low lymphocyte content in BALF, but with progression of lung changes.

Exhaled breath condensate 8-isoprostane, although increased in sarcoidosis, is not only unrelated to the production of superoxide anion by BALF cells, but seems to show reverse trends in relation to percentage of lymphocytes. The highest levels have been found in patients with radiological stage III. In our previous study [12], we found that increased EBC 8-isoprostane in sarcoid patients was not correlated with BALF lymphocytes, but were correlated with BALF eosinophils, and BALF eosinophils were inversely correlated with DLCO. The present study, dedicated to the mutual relationship between the momentary production of superoxide anion by activated BALF cells and 8-isoprostane in EBC, seems to confirm our earlier observations. These results are also consistent with the results of Montuschi et al. [10], who found that BAL 8-isoprostane in sarcoidosis negatively correlates with BAL lymphocytes.

We provided BAL 8-isoprostane results, but only for the estimation of correlations. We did not find any between BAL 8-isoprostane and superoxide production by BAL cells. We confirmed our earlier findings of a positive correlation between EBC and BAL 8-isoprostane [12]. Moreover, weak but negative correlations between BAL 8-isoprostane and FVC percent predicted and FEV₁/FVC ratio confirm our main results.

The lack of BAL 8-isoprostane results for the control group is an important limitation of the present study. The main reason was the lack of consent from the healthy volunteers (EBC control group) to be subjected to bronchoscopy. On the other hand, the control group for BAL, although consisting of healthy subjects, is not an "ideal" control group for EBC. As the primary objective of the study was the estimation of the correlations between exhaled breath 8-isoprostane and superoxide generation, we decided to approve this disadvantage.

One of the limitations of the use of 8-isoprostane in the monitoring of sarcoidosis and other lung diseases is a wide range of results in a study group and a substantial overlapping with control group results. One of the factors that could be responsible for this finding may be different antioxidant potential of individual subjects, which may be

genetically determined [25]. 8-Isoprostane is not a specific marker for sarcoidosis, therefore other inflammatory diseases may influence the results. Certainly, both the control and study groups were strictly selected towards exclusion criteria, such as infection, atopy and other inflammatory diseases of the respiratory and digestive tracts, but some subclinical states cannot be excluded with 100% certainty.

The two most likely explanations of the discrepancy between EBC 8-isoprostane and superoxide production by BAL cells are the following:

First, that superoxide anion and other ROS may be scavenged immediately after formation by naturally occurring antioxidants, preventing further peroxidation of membrane phospholipids. Although antioxidant protection was not analyzed in this paper, this possibility should be tested in a separate study.

Second, that other than alveolar inflammatory cells, sources of ROS may play a role in initiation of lipid peroxidation. For instance, alveolar epithelial cells covering a huge area of alveoli are potent sources of reactive oxygen and nitrogen species [26, 27].

The overall conclusion is that 8-isoprostane and superoxide generation by BAL cells characterize different processes. The latter is related to the intensity of inflammation, and is more common in less severe forms. 8-Isoprostane, related to peroxidation of lipid membranes, may describe chronic destruction linked to more chronic forms and worse prognosis. The latter statement deserves further follow-up studies for confirmation of the prognostic value of EBC 8-isoprostane.

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