



## Hydrogen Peroxide in Expired Air Condensate Correlates Positively with Early Steps of Peripheral Neutrophil Activation in Asthmatic Patients

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**Abstract.** We have found an increased  $\text{H}_2\text{O}_2$  level in expired air of asthmatic patients. Neutrophils from these subjects generated higher amounts of superoxide radicals after challenge with phorbol esters than those from healthy subjects which may result from an increased activity of NADPH-oxidase. The enhanced  $\text{Ca}^{2+}$  mobilisation in neutrophils from asthmatics could be responsible for increased production and subsequent elevated  $\text{H}_2\text{O}_2$  concentration in expired breath condensate. In this study we wished to determine whether neutrophils of asthmatic patients have enhanced  $[\text{Ca}^{2+}]_i$  response after N-formyl-methionyl-leucyl-phenylalanine – fMLP challenge as compared with cells from healthy donors, and if so, does it correlate with  $\text{H}_2\text{O}_2$  levels in expired air. We examined 21 patients, 10 healthy individuals as a control group (mean age  $34.3 \pm 5.5$ , 6 males and 4 females) and 11 asthmatic subjects (mean age  $38.2 \pm 7.2$ , 7 males and 4 females). The rise of  $[\text{Ca}^{2+}]_i$  as an early event of neutrophil activation, was measured spectrofluorimetrically with Fura-2-AM. The mean  $\text{H}_2\text{O}_2$  level, measured spectrofluorimetrically in the expired breath of asthmatics, was 20-fold higher than that in healthy control ( $0.18 \pm 0.20$  vs.  $0.01 \pm 0.04 \mu\text{M}$ ,  $p < 0.05$ ).  $[\text{Ca}^{2+}]_i$  increase after challenge by fMLP ( $\Delta[\text{Ca}^{2+}]_i$ ) was much higher in asthmatics than in control group ( $205.0 \pm 44$  vs.  $113.0 \pm 22$  nM,  $p < 0.05$ , respectively). A strong correlation was observed between  $\text{H}_2\text{O}_2$  and  $\Delta[\text{Ca}^{2+}]_i$  and maximal velocity of increase in  $[\text{Ca}^{2+}]_i$  in asthmatics ( $r = 0.87$ ,  $p < 0.01$  and  $r = 0.64$ ,  $p < 0.05$ ). We conclude that elevated  $\text{H}_2\text{O}_2$  level in the expired breath condensate of asthmatics can be generated by activated neutrophils in the course of mucosal inflammation observed in bronchial asthma.

**Key words:** bronchial asthma; hydrogen peroxide in breath condensate; neutrophils; intracellular calcium.

### Introduction

In several lung inflammatory diseases an elevated hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content in the expired breath has been found, among which bronchial asthma seems to be extensively studied<sup>3, 15, 28, 33, 41</sup>.  $\text{H}_2\text{O}_2$  is one of the most stable, toxic oxygen metabolites and relatively easy to detect. It is also volatile, and due to lack of charge membrane permeable. It is cytotoxic<sup>38</sup>, a high concentrations  $\text{H}_2\text{O}_2$  causes cell necrosis and at low levels can induce apoptosis<sup>16</sup>. Catalase and glutathione

peroxidase are basic enzymes regulating intracellular  $\text{H}_2\text{O}_2$  level<sup>11, 35</sup>. Moreover, in asthmatic patients increased level of  $\text{H}_2\text{O}_2$  positively correlated with enhanced level of lipid peroxidation products (thiobarbituric acid reactive species) in expired breath condensate which proves oxidant/antioxidant imbalance in the airways of these patients<sup>3</sup>. An intense airway inflammation can be caused either by  $\text{H}_2\text{O}_2$  alone or newly generated hydroxyl radical ( $\text{OH}^\bullet$ )<sup>4</sup>. An important feature of bronchial asthma is the influx of circulating phagocytes including eosinophils, mast cells

and neutrophils into the bronchial wall<sup>26</sup>. When activated, they are capable of generation of reactive oxygen species, for example superoxide anion ( $O_2^{\cdot-}$ ) which is then dismutated to hydrogen peroxide ( $H_2O_2$ ) and can play an important role in the development of pathophysiological features of bronchial asthma, like enhanced arachidonic acid release, smooth muscle contraction, impaired  $\beta$ -adrenergic responsiveness and bronchial hyperresponsiveness<sup>1, 4, 22</sup>. It was proved that neutrophils from both adult and children asthmatic patients can higher increase amounts of  $O_2^{\cdot-}$  and  $H_2O_2$  after challenge with phorbol esters and fMLP than cells on matched, healthy subjects<sup>21, 23, 40</sup>. Moreover, the ability of neutrophils isolated from asthmatics to produce superoxide anion, correlated with the degree of airway hyperresponsiveness to inhaled metacholine and histamine<sup>19, 21, 25</sup>. A close relationship between the opsonized zymosan-induced chemiluminescence of neutrophils and bronchial hyperreactivity to histamine in asthmatic children can also be observed<sup>24</sup>. Furthermore, in the same asthmatic children, the generation of  $O_2^{\cdot-}$  was significantly higher with than without asthma attacks. This is consistent with the observation that exacerbated asthma patients exhale more  $H_2O_2$  than patients with stable disease<sup>15, 28</sup>. Similarly, an enhanced alveolar cell luminol-dependent chemiluminescence in asthmatic patients has been also found<sup>12</sup>.

An important source of  $H_2O_2$  are phagocytes due to NADPH oxidase system generating at least 3 oxygen metabolites ( $O_2^{\cdot-}$ ,  $H_2O_2$  and  $OH^{\cdot}$ )<sup>6, 30</sup>. They are released in extracellular fluid, and in the airways a part of  $H_2O_2$ , which has not been decomposed by antioxidant enzymes, can be excreted with expired air. NADPH oxidase system is activated by a variety of stimuli including: 1,2-diacylglycerol, phosphatidic acid, inositol triphosphate and  $Ca^{2+}$ <sup>31, 39</sup>. Changes of intracellular free calcium concentration  $[Ca^{2+}]_i$  seem to be an important early step in signal transduction leading to the respiratory burst and degranulation. Increased  $[Ca^{2+}]_i$  promotes diacylglycerol-induced activation of protein kinase C that is responsible for phosphorylation of cytoplasmic subunits of NADPH oxidase. Neutrophils depleted with  $Ca^{2+}$  fail to produce superoxide in response to stimulation unless they are supplied with extracellular  $Ca^{2+}$ <sup>32</sup>. Moreover, activation of the NADPH oxidase occurs when cytosolic  $Ca^{2+}$  rises<sup>18</sup>. What is very interesting, an increased release of calcium intracellular stores in neutrophils, obtained from asthmatic patients after stimulation with fMLP compared with healthy subjects was observed<sup>5</sup>. A hypothesis, therefore, can be put forward that increased production of reactive oxygen species by neutrophils from asthmatic patients

may be caused by enhanced  $Ca^{2+}$  mobilization. This would lead to an increased  $H_2O_2$  generation which could evaporate from the alveolar lining fluid and be detected in expired air. In this study we found a correlation between  $[Ca^{2+}]_i$  rises, as an early step of neutrophil activation after fMLP challenge, and  $H_2O_2$  in expired air of asthmatic patients. This could reflect  $Ca^{2+}$  involvement in the generation of reactive oxygen species as inflammatory mediators in the course of bronchial asthma.

## Materials and Methods

**Reagents.** Peroxidase from horseradish type II (HRP, 200 U/mg solid), homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid), and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were from Sigma Chemicals Co. (St Louis, MO, USA). Glycine, ethylene diamine tetra-acetic acid (EDTA), phosphate buffered saline (PBS, pH 7.4) and 30%  $H_2O_2$  solution, glacial acetic acid,  $CaCl_2$ , KCl, and NaCl were purchased from POCH (Gliwice, Poland). Fura-2-AM (acetoxymethyl ester), ethylene glycol bis (2-aminoethyl-ether)-tetraacetic acid (EGTA), Triton X-100, dimethyl sulphoxide (DMSO) were from Serva (Heidelberg, Germany).  $H_2O_2$  solution (30%) was diluted 100-fold with PBS and stored at 4°C in the dark. The actual  $H_2O_2$  concentration was calculated from its absorbance at 230 nm ( $E = 81 \text{ cm}^{-1}\text{M}^{-1}$ ). Aqueous solution of 1 U/ml HRP with addition of 400  $\mu\text{M}$  homovanillic acid were prepared freshly before the assay. FMLP, Fura-2-AM were dissolved in DMSO to final concentration 60  $\mu\text{M}$  and 2 mM, respectively, and stored at -80°C until assay. All solutions were stored at 4°C not longer than for 14 days.

**Study population.** The study included 10 healthy volunteers as a control group (mean age  $34.3 \pm 5.5$  year, 6 males and 4 females) and 11 asthmatic subjects (mean age  $38.2 \pm 7.4$  year, 7 males and 4 females) who

**Table 1.** Characteristic of study population

|                        | Healthy subjects | Asthmatic patients |
|------------------------|------------------|--------------------|
| Number                 | 10               | 11                 |
| Age (year)             | $34.3 \pm 5.5$   | $38.2 \pm 7.4$     |
| Sex M: F               | 6:4              | 7:4                |
| FVC%                   | $96.5 \pm 1.2$   | $92.5 \pm 6.8$     |
| FEV1%                  | $101.5 \pm 4.7$  | $67.1 \pm 4.2^*$   |
| PEFR%                  | $101.3 \pm 5.6$  | $68.1 \pm 13.1^*$  |
| FEV1 reversibility (%) | $5.05 \pm 3.3$   | $30.2 \pm 48.8^*$  |
| Asthma duration (year) | —                | $7.0 \pm 5.0$      |

Significantly different from pulmonary function tests of healthy subjects: \*  $p < 0.001$ .

had not suffered from any infectious disease for the last 4 months (Table 1). They were recruited from University Medical School out-patient clinic register. Asthmatic subjects were asked to stop any medication except short-acting  $\beta$ -agonists (salbutamol or fenoterol) and to report to the clinic after 4-week washout period to perform lung function tests. Only those patients who were able to refrain from antiinflammatory medication, indicated in this group, during washout were included to the study. Bronchial asthma was diagnosed based on history of wheezing dyspnea and previous documentation of bronchodilator-induced bronchial reversibility measured as more than 15% increase of FEV1 and the presence of airway hyperreactivity after histamine challenge test with PC20 of less than 8 mg/ml according to the method of COCKROFT<sup>13</sup>.

Bronchial reversibility at least 15% of the baseline and the ability to stop other than  $\beta$ -agonist therapy were basic inclusion criteria. The duration of bronchial asthma was 1 to 18 years, mean  $7 \pm 5$  years. Spirometry was performed with FlowScreen (Erich Jaeger GmbH&Co., Germany) equipped with software compatible to American Thoracic Society standards<sup>2</sup>. This study was approved by the local Ethics Committee and informed consent was obtained.

**Collection of air condensate.** The air condensate was collected in a tube installed in the polystyrene foamed container filled with ice and salt as previously described<sup>33</sup>. Briefly, study subjects were asked to breathe into the apparatus and then the condensate was transferred to Eppendorf tubes. They were stored at  $-80^{\circ}\text{C}$  for not longer than 7 days until  $\text{H}_2\text{O}_2$  measurement. Our previous experiments have shown that samples of expired breath condensate and  $10^{-7}$  M  $\text{H}_2\text{O}_2$  solution, under these conditions, remain stable after 14 days of storage. Similarly, 50 nM  $\text{H}_2\text{O}_2$  incubated in the device for 20 min at  $0^{\circ}\text{C}$ , revealed no significant changes of the ability to react with homovanillic acid<sup>12</sup>. All collections were performed between 9 and 11 a.m. and patients were asked to stop any medication 12 h before the visit.

**Measurement of hydrogen peroxide.** The content of  $\text{H}_2\text{O}_2$  in expired breath condensate was determined according to the method of RUCH et al.<sup>36</sup>. Briefly, 600  $\mu\text{l}$  of expired breath condensate was mixed with 600  $\mu\text{l}$  HRP solution of (1 U/ml) containing 100  $\mu\text{M}$  homovanillic acid and incubated for 60 min at  $37^{\circ}\text{C}$ . Afterwards, the sample was mixed with 150  $\mu\text{l}$  0.1 M glycine-NaOH buffer (pH 12) with addition of 25 mM EDTA and transferred into microcuvette (PE 5200-4339). The homovanillic acid oxidation product as a measure of the amount of  $\text{H}_2\text{O}_2$  was determined spec-

trofluorimetrically using Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT) operating in the read mode. Slit widths were set at 10 nm for both emission and excitation and the integrate time was 0.1 s. Excitation was at 312 nm and emission was measured at 420 nm. Readings were converted into nM using regression equation:

$$Y = 67.64 (X - X_0),$$

where  $Y$  – nmol of  $\text{H}_2\text{O}_2$  per one litre of expired breath condensate,  $X$  – intensity of emission at 420 nm expressed in arbitrary units,  $X_0$  – intensity of emission given by reference sample receiving distilled water instead of breath condensate, obtained from 3 series of calibration experiments with 19 increasing (0.0125 to 25  $\mu\text{M}$ )  $\text{H}_2\text{O}_2$  concentrations. The confidence level was 95% and  $p$  value was less than 0.03 and 0.0001 for constant and regression coefficient, respectively. The linear least square estimation was used for calculation of the regression equation. The lower limit of  $\text{H}_2\text{O}_2$  detection was 83 nM and the calibration curve was linear up to a concentration 16.7  $\mu\text{M}$   $\text{H}_2\text{O}_2$  concentrations.

**Cell preparation.** A 20 ml sample of the whole blood was obtained from all subjects included in the study between 8 and 9 a.m. Human neutrophils were isolated by dextran sedimentation followed by centrifugation by Ficoll-Hypaque according to standard procedures<sup>8</sup>. The viability of neutrophil suspensions was always above 96% and the purity was 98% as assessed by trypan blue exclusion test and analysis of smears prepared from each sample using Giemsa stain, respectively.

**Fura-2-AM loading and measurement of cytosolic free calcium.** Neutrophil  $\text{Ca}^{2+}$  response was measured as previously described<sup>34</sup>. Briefly, human neutrophils ( $5 \times 10^6/\text{ml}$ ) were suspended in 6 mM Tris-HCl buffer (pH 7.4, osmolality 283 mOsm/kg  $\text{H}_2\text{O}$ ). Cells were incubated with 1  $\mu\text{M}$  Fura-2-AM for 1 h at  $37^{\circ}\text{C}$  in total darkness in atmosphere containing 5%  $\text{CO}_2$ <sup>17</sup>. After 2 washes with buffered saline cells were resuspended ( $2 \times 10^6/\text{ml}$ ) in 6 mM Tris-HCl buffer (pH 7.4, osmolality 312 mOsm/kg  $\text{H}_2\text{O}$ ) and incubated for 15 min at  $37^{\circ}\text{C}$ . During all experiments cells were kept at  $0^{\circ}\text{C}$  in darkness for not more than 2 h to prevent the leakage of Fura-2 and warmed up to  $37^{\circ}\text{C}$  just before use. Neutrophils were stimulated by addition of 1  $\mu\text{l}$  of fMLP solution (final concentration  $10^{-7}$  M).  $[\text{Ca}^{2+}]_i$  was determined by dual wavelength measurements at emission wavelength of 510 nm and excitation wavelength of 340 nm and 380 nm in the Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT). Slit

widths were set at 5.0 nm for emission and at 10.0 nm for excitation. All measurements were performed at 37°C under constant stirring of 600 µl of neutrophil suspension ( $1.2 \times 10^6$  cells) kept in microcuvette (PE 5200-4339). Fluorescence signals were calibrated after lysis of neutrophils with 0.1% Triton X-100 (maximal signal) and subsequent addition of EGTA to a final concentration of 10 nM (minimal signal). Preliminary experiments have shown that 0.1% Triton X-100 had not changed the 340/380 nm ratio. Autofluorescence of neutrophils was determined with cells loaded with DMSO alone. All operations and calculations of  $[Ca^{2+}]_i$  were performed with use of "The Intracellular Biochemistry Application" software (Perkin Elmer, Beaconsfield, England 1990).

**Statistical analysis.**  $H_2O_2$  concentration was expressed as the mean  $\pm$  SD. For readings that gave results below the limit of sensitivity,  $H_2O_2$  concentration in expired breath condensate were assumed as 0 nM. The following parameters of neutrophil response were determined and also expressed as mean value  $\pm$  SD:  $\Delta[Ca^{2+}]_i$  – maximal increment in the  $[Ca^{2+}]_i$ ,  $V$  – maximal velocity of the increase in  $[Ca^{2+}]_i$ ,  $m[Ca^{2+}]_i$  – maximal  $[Ca^{2+}]_i$  after activation,  $\Delta t$  – time to reach  $m[Ca^{2+}]_i$ . The differences between results in the groups of healthy and asthmatic subjects were determined by the Student's *t*-test. A *p* value less than 0.05 was considered to be significant. Pearson correlation was used to determine the relations between measured variables. All calculations were performed using Microsoft Excel version 5.0 software.

## Results

Our previous paper on  $H_2O_2$  levels in the air condensate from the same subject revealed that  $H_2O_2$  concentration in expired breath remains stable for 4-day observation and that there are no significant differences in separate samples collected on the same day with 30 min intervals<sup>33</sup>. If patients did not rinse their mouths with distilled water before and during condensation,  $H_2O_2$  level rose which was due to the saliva contamination. It was proven by SZNAJDER et al.<sup>41</sup> that  $H_2O_2$  level in saliva is 7-fold higher than that in the expired air. So in all experiments on the content of  $H_2O_2$  in expired breath condensate the noseclip was used and rinsing mouth with distilled water was performed.

Only one healthy volunteer (1 male) revealed detectable  $H_2O_2$  content in the air condensate (0.18 µM). In 9 healthy subjects the  $H_2O_2$  level was below the method sensitivity (83 nM) and were assumed as 0 nM. This is why the mean  $H_2O_2$  concentration calculated for

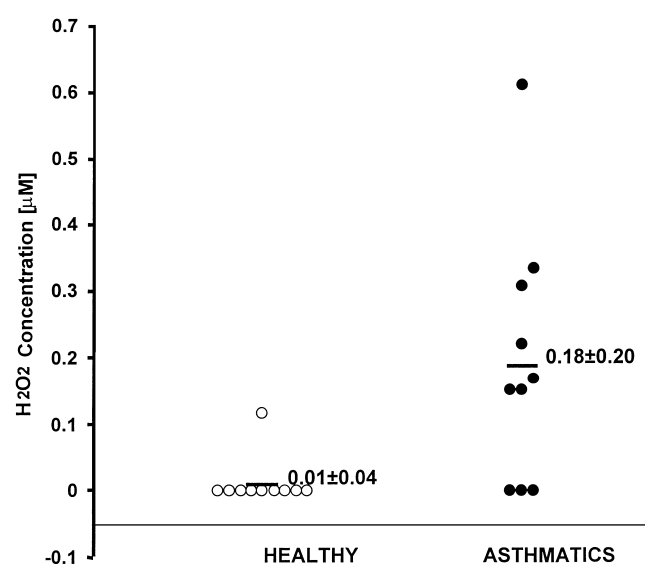
**Table 2.** Parameters of fMLP-induced  $Ca^{2+}$  response of neutrophils from healthy volunteers and asthmatic patients

| Subject    | Parameters of fMLP-induced neutrophil $Ca^{2+}$ response |                        |                   |
|------------|----------------------------------------------------------|------------------------|-------------------|
|            | $\Delta [Ca^{2+}]_i$<br>(nM)                             | $m[Ca^{2+}]_i$<br>(nM) | $V$<br>(nM/s)     |
| Healthy    | $113.9 \pm 22.3$                                         | $205.0 \pm 44.7$       | $8.38 \pm 2.23$   |
| Asthmatics | $177.8 \pm 21.7^*$                                       | $292.8 \pm 60.8^*$     | $4.23 \pm 1.33^*$ |

$\Delta [Ca^{2+}]_i$  – maximal increment in  $[Ca^{2+}]_i$ ;  $m[Ca^{2+}]_i$  – maximal  $[Ca^{2+}]_i$  after activation,  $V$  – maximal velocity of the increase in  $[Ca^{2+}]_i$  after stimulation with  $10^{-7}$  M fMLP.

Significantly different from neutrophils from healthy donors:

\**p* < 0.001.

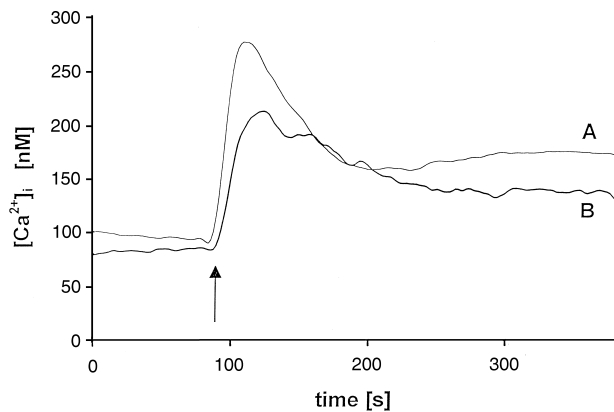


**Fig. 1.**  $H_2O_2$  concentration in expired breath condensate of asthmatic patients and healthy subjects. Individual results below the sensitivity of  $H_2O_2$  method determination (83 nM) were assumed as 0 nM. The mean  $H_2O_2$  content of breath condensate of all asthmatic subjects was higher than that found in control group  $0.18 \pm 0.20$  µM vs.  $0.01 \pm 0.04$  µM (*p* < 0.05)

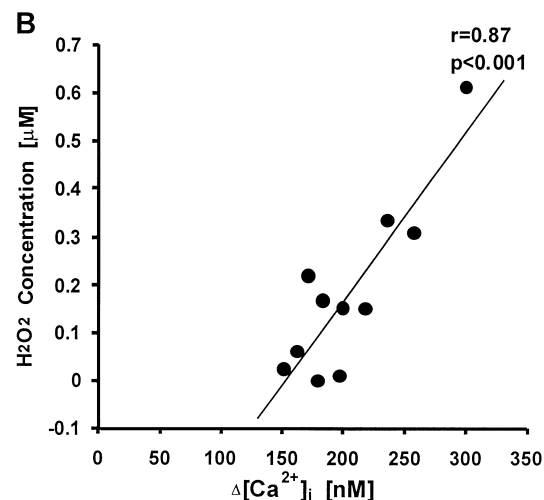
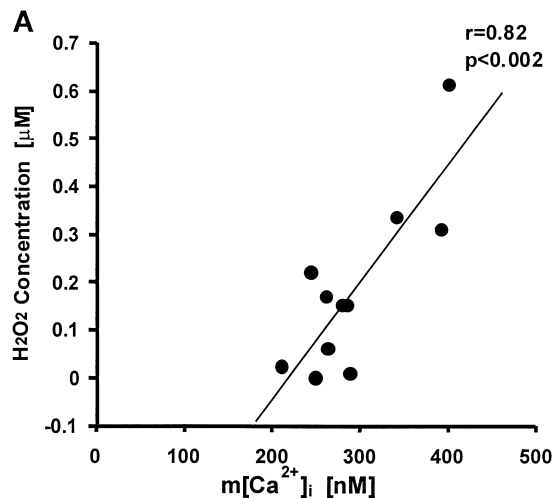
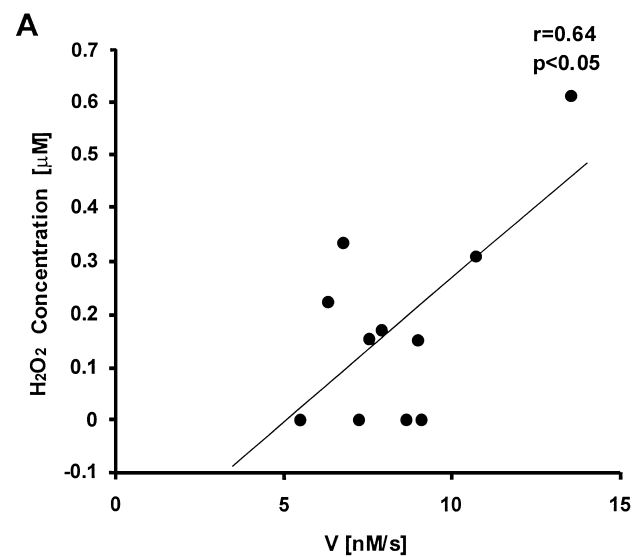
whole group was  $0.01 \pm 0.04$  µM. The  $H_2O_2$  level in expired breath condensate of asthmatic subjects (*n* = 11) was almost 18-fold higher than that in control group and was  $0.18 \pm 0.20$  µM (*p* < 0.05) (Fig. 1). In 1 female asthmatic no detectable  $H_2O_2$  concentration was noted.

There were no significant differences between male and female asthmatic subjects as far as  $H_2O_2$  level is concerned.

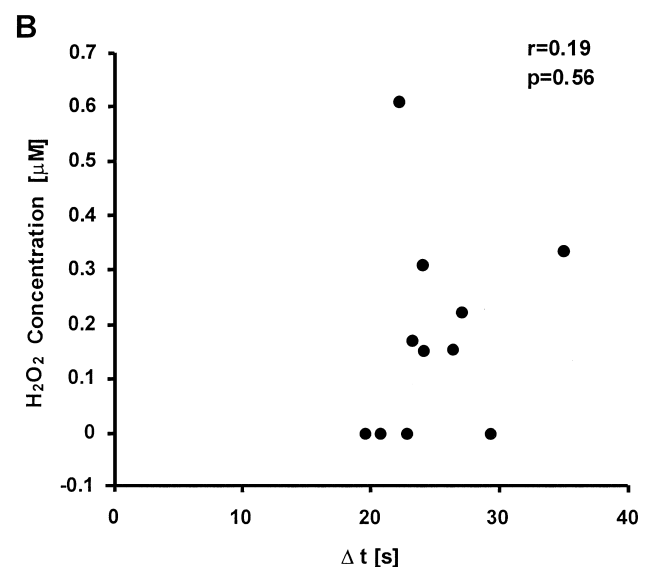
Both, neutrophils from healthy and asthmatic patients responded to fMLP stimulation with increased their  $[Ca^{2+}]_i$  (Fig. 2). The  $Ca^{2+}$  response was higher in neutrophils from asthmatic than from healthy donors. Maximal increment in  $[Ca^{2+}]_i$  ( $\Delta[Ca^{2+}]_i$ ) and maximal  $[Ca^{2+}]_i$  after stimulation ( $m[Ca^{2+}]_i$ ) were 2-fold and



**Fig. 2.** Mean changes of  $[Ca^{2+}]_i$  in Fura-2 loaded human PMNL after stimulation with  $10^{-7}$  MfMLP. Cells from asthmatic subjects (line A) and from healthy volunteers (line B) were suspended in 1 mM  $Ca^{2+}$  buffer. Arrow indicates time of agonist challenge



**Fig. 3.** Significant positive correlation between  $H_2O_2$  concentration in expired air condensate and (A) maximal  $[Ca^{2+}]_i$  after activation ( $m[Ca^{2+}]_i$ ) or (B)  $\Delta[Ca^{2+}]_i$  in PMNL of asthmatic patients after stimulation with fMLP ( $r = 0.82$ ,  $p < 0.002$ ;  $r = 0.87$ ,  $p < 0.001$ , respectively)



**Fig. 4.** Significant positive correlation between  $H_2O_2$  concentration in expired air condensate and (A) maximal velocity of the increase in  $[Ca^{2+}]_i$  ( $V$  [nM/s]) or no correlation with (B) time to reach  $m[Ca^{2+}]_i$  ( $\Delta t$  [s]) in PMNL of asthmatic patients after stimulation with fMLP ( $r = 0.64$ ,  $p < 0.05$ ;  $r = 0.19$ ,  $p = 0.56$ , respectively)

1.6-fold higher in neutrophils from asthmatic than from healthy subjects ( $205.0 \pm 44.7$  vs.  $113.9 \pm 22.3$ , and  $292.8 \pm 60.8$  vs.  $177.8 \pm 21.7$  nM,  $p < 0.001$  and  $p < 0.001$ , respectively) (Table 2). Neutrophils from asthmatics revealed also a much higher maximal velocity of the increase in  $[Ca^{2+}]_i$  ( $V$ ) as compared with healthy donors ( $8.4 \pm 2.0$  vs.  $4.0 \pm 1.0$  nM/s, respectively,  $p < 0.001$ ) (Table 2). There was no significant difference between study groups in the time ( $\Delta t$ ) to reach  $m[Ca^{2+}]_i$ .

What is interesting, a positive correlation was found

between  $\text{H}_2\text{O}_2$  in expired breath condensate and the early step of fMLP-induced neutrophil activation expressed as both  $\Delta[\text{Ca}^{2+}]_i$  and  $m[\text{Ca}^{2+}]_i$  in neutrophils of asthmatic patients ( $r = 0.87$ ,  $p < 0.001$ , and  $r = 0.82$  and  $p < 0.002$ ) (Fig. 3), respectively. There was also a positive correlation between  $\text{H}_2\text{O}_2$  in expired breath condensate and maximal velocity of the increase in  $[\text{Ca}^{2+}]_i$  (V) in neutrophils of asthmatic patients ( $r = 0.64$ ,  $p < 0.05$ ) but no correlation between  $\text{H}_2\text{O}_2$  in expired air and  $\Delta t$  ( $r = 0.19$ ,  $p = 0.56$ ) (Fig. 4), respectively. No correlation was found between measured parameters in healthy subjects.

## Discussion

In this study we have found that asthmatic subjects have higher  $\text{H}_2\text{O}_2$  level in expired breath condensate as compared with healthy volunteers and that there is a strong positive correlation between  $\text{H}_2\text{O}_2$  in expired air and neutrophil  $\text{Ca}^{2+}$  response after fMLP challenge. It seems quite likely that the inflammatory processes in the respiratory tract lead to increased oxidant production, which in turn can be detected by elevated  $\text{H}_2\text{O}_2$  in expired breath. Thus  $\text{H}_2\text{O}_2$  could reflect the presence of airway inflammation. This hypothesis seems to be supported by the fact that  $\text{H}_2\text{O}_2$  concentrations are lower in asthmatics who use antiinflammatory medication<sup>20</sup>.

Increased levels of  $\text{H}_2\text{O}_2$  in expired breath were found not only in bronchial asthma. Our previous reports have shown that healthy cigarette smokers have increased  $\text{H}_2\text{O}_2$  in expired breath<sup>33</sup>. SZNAJDER et al.<sup>41</sup> have found increased  $\text{H}_2\text{O}_2$  levels measured in the breath condensate of patients with ARDS. DOHLMAN et al.<sup>15</sup> revealed relatively high  $\text{H}_2\text{O}_2$  levels in expired breath condensate in pediatric patients with asthma. Increased  $\text{H}_2\text{O}_2$  in expired air of asthmatic children was also found by JOBSIS et al.<sup>20</sup>. Moreover, they also showed that antiinflammatory therapy, as inhaled steroids, is associated with a lower exhaled  $\text{H}_2\text{O}_2$  concentration.

Increased content of  $\text{H}_2\text{O}_2$  in expired breath condensate of asthmatic subjects is likely to be due to increased oxidant production in bronchial lining fluid and the overcome antioxidant potential of lower airways. Increased  $\text{H}_2\text{O}_2$  in expired breath condensate of asthmatics can reflect a situation in which oxidant overload overtakes the capacity of antioxidant protection in the lower airways.

Increased number of polymorphonuclear cells (mainly eosinophils, but also neutrophils and macrophages) have been identified at autopsy of patients dying of asthma<sup>14</sup> or by use of BAL<sup>7</sup> and induced spu-

tum<sup>27</sup>. The most important source of  $\text{H}_2\text{O}_2$  seems to be eosinophils. Differential cell counts showed significant eosinophil percentage in induced sputum as compared with controls<sup>27</sup>. On the other hand, neutrophils seem to be quite active in the airways of asthmatics. Significantly higher concentrations of neutrophil granule proteins (myeloperoxidase and human neutrophil lipocalin) in induced sputum of asthmatic patients were found<sup>27</sup>. This finding indicates that neutrophils in the airways of asthmatic patients are actively degranulated and it is likely that these proteins and eosinophil granule proteins, as major basic proteins, contribute to the airway damage seen in asthma<sup>29</sup>. Neutrophils can be primed and/or activated by several cytokines including IL-4, IL-8, and GM-CSF. These cytokines activate intracellular tyrosine kinases leading to phosphorylation of several intracellular proteins including those involved in  $\text{Ca}^{2+}$  balance and subunits of NADPH oxidase<sup>9</sup>. Phosphorylation of these proteins may lead to enhanced oxygen reactive species production by PMNL from asthmatic patients and increased hydrogen peroxide exhalation.

We have demonstrated that neutrophils from asthmatic patients responded with higher  $[\text{Ca}^{2+}]_i$  rise after stimulation with fMLP than control cells. The enhanced response to fMLP may come from increased number of fMLP receptors on neutrophil outer membrane and/or altered intracellular signal transduction expressed by enhanced inositol triphosphate generation opening calcium channels of intracellular  $\text{Ca}^{2+}$  stores. Neutrophils from asthmatics can be primed continuously and this is why ready to react so easily. Higher intracellular  $\text{Ca}^{2+}$  accumulation in neutrophils of asthmatic patients should be also taken into account. Increased  $[\text{Ca}^{2+}]_i$  responses in asthmatics may be an explanation of these phenomena. As our patients refrained from any medication (except short acting  $\beta$ -agonists 6 h before blood collection) especially any steroids for 3 months before entering the study, any inhibiting steroid action on mediator release from inflammatory cells should be excluded.

It is very interesting that a strong positive correlation was found between  $[\text{Ca}^{2+}]_i$  response after fMLP challenge and  $\text{H}_2\text{O}_2$  concentration in exhaled air of asthmatic patients. As we noted before, increased  $[\text{Ca}^{2+}]_i$  is one of the intracellular triggers of NADPH oxidase. The correlation found could reflect a cause-and-effect relationship. Increased  $[\text{Ca}^{2+}]_i$  in neutrophils of asthmatic patients may trigger enhanced reactive oxygen species production and thus be responsible for enhanced  $\text{H}_2\text{O}_2$  level in expired breath condensate of these patients. Obviously, there can be other sources of  $\text{H}_2\text{O}_2$

in the airways of asthmatic patients i. e. macrophages or eosinophils and their role in the free radical generation in asthma remains to be fully explained<sup>10, 37</sup>.

Our results indicate that asthmatic patients exhale more H<sub>2</sub>O<sub>2</sub> and that this phenomenon seems to be tightly associated with [Ca<sup>2+</sup>]<sub>i</sub> increase which is an early event of the activation of neutrophils of these patients which proves phagocyte involvement in the development of airway oxidant overburden in bronchial asthma.

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