Expired hydrogen peroxide in breath condensate of cystic fibrosis patients

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Expired hydrogen peroxide in breath condensate of cystic fibrosis patients. L.P. Ho, J. Faccenda, J.A. Innes, A.P Greening. ©ERS Journals Ltd 1999.
ABSTRACT: Stimulated inflammatory cells release large amounts of hydrogen peroxide (H_2O_2). Breath condensate H_2O_2 has been shown to be elevated in stable asthmatic children, chronic obstructive pulmonary disease and intubated adult respiratory distress syndrome. In cystic fibrosis airways, where neutrophilic inflammation dominates, it is postulated that H_2O_2 in breath condensate would be elevated and may be used as a marker of airways inflammation.

Expired breath condensate was collected from 16 clinically stable cystic fibrosis (CF) patients (mean age 25.3 yrs, mean forced expiratory volume in one second (FEV_1) 50.2%) and 14 normal subjects (mean age 29.9 yrs). Total plasma leukocyte, neutrophil, monocyte and eosinophil counts and lung function were also measured on the day of collection. A method of breath condensate collection excluding the confounding factors of nasal air and saliva contamination was validated and used and H_2O_2 measured fluorometrically using an optimized assay.

The median level of H_2O_2 concentration in breath condensate of CF patients was lower than that in normal subjects (0.064 *versus* 0.089 µM), but this did not reach statistical significance (p=0.20, Mann–Whitney rank sum test). Within the CF group, there was no correlation between H_2O_2 concentration and lung function.

Expired breath condensate H_2O_2 is not elevated in patients with cystic fibrosis, and is thus not a suitable marker of airways inflammation in these patients. Possible explanations include physical barriers to its detection caused by viscous airways secretions, reaction with other reactive species or increased antioxidant activity caused by trapping of positively charged antioxidants in negatively charged airways secretions.
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Lung disease in cystic fibrosis (CF) is characterized by chronic bacterial colonization and airways suppuration, with progressive destruction of the airways resulting in respiratory failure. Lung injury in CF patients has been attributed to various factors, although the basic mechanisms have not been fully elucidated. Abnormal airways secretions, decreased mucociliary clearance and loss of activity of innate antibacterial molecules like human-β defensins [1] generate a permissive environment for bacterial colonization and infection, driving chronic inflammation.

Airway inflammation plays a central role in the initiation and pathogenesis of end stage respiratory failure in CF. Expression of pro-inflammatory cytokines like interleukin (IL)-8 is upregulated in CF airways epithelium even before the onset of bacterial colonization [2] and CF broncho-alveolar lavage fluid contains high levels of inflammatory cells, particularly neutrophils, and pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α [3, 4]. The major contribution to cell damage is thought to be caused by the proteolytic and oxidative products released by these inflammatory cells. The primary reactive oxygen metabolites formed *in vivo* are superoxide and hydrogen peroxide (H_2O_2), released during oxidative bursts associated with sequestration of invading organisms. H_2O_2 is also generated through nonenzymatic and enzymatic dismutation of superoxide. Owing to the lability of superoxides, direct detection

is difficult [5], but H_2O_2 is volatile, more stable and enters the gaseous phase at physiological temperatures [6]. It can thus be measured by fluorometric assays which utilize the oxidation of a substrate to a fluorescing compound by H_2O_2. With this method, H_2O_2 has been found to be elevated in breath condensate of patients with chronic obstructive pulmonary disease (COPD), asthma and adult respiratory distress syndrome (ARDS) [7–11] and has been suggested to represent the oxidant burden in these conditions.

It was postulated that H_2O_2 would be elevated in patients with CF due to the increased level of neutrophilic inflammation and activated macrophages and that H_2O_2 in the condensed breath may be used to follow airways inflammation in these patients. In this study the levels of H_2O_2 were measured in breath condensate of CF patients and normal healthy controls using a modified assay which allowed detection of low levels of H_2O_2 and examined if this is correlated to lung function and circulating plasma leukocytes.

Methods

Patients

Sixteen clinically stable CF patients and 14 normal subjects were recruited (table 1). Lung function in the CF subjects ranged 14.8–77.8% of predicted forced expiratory

Table 1. – Demographic data of the study groups

	Cystic fibrosis patients (n=16)	Normal subjects (n=14)
Mean±SD age yrs	25.3±6.5	29.9±6.2
Sex M:F	9:7	6:8
Mean±SD FEV1 %	50.2±20.1	Not done

Data are mean SD. M: male; F: female; FEV1: forced expiratory volume in one second.

volume in one second (FEV1) (mean FEV1: 50.2%) but all were clinically stable at time of sampling. A "clinically stable" patient was defined as one with no change to the severity of clinical symptoms (cough, sputum production, malaise and dyspnoea) over the 2 months before sampling and where the FEV1 at the time of sampling was not different by >20% compared with the average value over the last 6 months. Eight of these patients showed reversible airways obstruction (increase in FEV1 of >175 mL after 2.5 mg nebulized salbutamol) and were on inhaled steroids during sampling. The dose of inhaled steroids ranged 400 µg beclomethasone·day^{-1} to 2000 µg fluticasone·day^{-1}. None of the patients were on recombinant deoxyribonuclease (DNase) or antibiotic treatment during sampling.

Normal subjects were healthy volunteers, with no evidence of active allergic rhinitis, concomitant respiratory tract infection or respiratory symptoms. For both groups, smokers were excluded. All subjects gave informed consent for the study and the study was approved by the local ethics committee.

Collection of breath condensate

Breath condensate was collected using a method where the subject inspired repeatedly to total lung capacity (TLC) and exhaled into 1.5 m Teflon perfluoralkoxy (PFA) tubing with 0.5 cm internal diameter, immersed in ice. This yielded 1 mL of breath condensate within 6–8 min. Subjects were instructed to maintain a dry mouth by periodically swallowing their saliva during the procedure. All samples were processed and assayed immediately after collection. Each patient contributed one sample. Within 2 h of breath collection, FEV1 was performed and venous blood collected for measurement of total circulating leukocyte count and differential analysis of leukocytes for neutrophils, monocytes and eosinophils.

Optimization of collection method

Since saliva- and nasal-derived air might contaminate the samples, the collection method was first tested to ensure exclusion of these factors. Breath condensate samples were collected in 15 CF subjects over 6 min and each tested for salivary amylase using a reflectrometric dry slide method with a Vitros analyser (Ortho Clinical Diagnostics, Strasbourg, France). Two samples were "spiked" with saliva to ensure that salivary contamination could be detected.

With regards to nasal air contamination, two subjects exhaled into the Teflon tube in the standard fashion while their noses were being flushed with 100% helium (BOC, Kent, UK) delivered by nasal cannulae. (The helium flushing started after expiration.) Expirate emerging from the

collecting tube was continuously sampled for helium detection during and for 15 s after the end of exhalation. In order to establish that this system could detect helium in the expirate, the subjects also inhaled to TLC while the nose was flushed with 100% helium and then exhaled into the collecting system where expirate was tested for helium as above.

Hydrogen peroxide assay

The concentration of H_2O_2 was quantified using a fluorometric assay based on the formation of an intermediate compound (compound I) from H_2O_2 by horseradish peroxidase which then oxidizes p-hydroxyphenyl acetic acid (PHAA) to a fluorogenic compound.

The method used by HYSLAR and SKLAR [12] was adapted and optimized according to factors described by RUCH et al. [13]. Briefly, a 50 mM solution of PHAA (Sigma-Aldrich, Poole, Dorset, UK) was added to 0.1 mg·mL^{-1} type II horse radish peroxidase (Sigma) in 1:3 (v:v) ratio on the study day. One millilitre of breath condensate was added to 1 mL of this solution within 5 min of collection and a pH of 9–10 achieved by the addition of 600 µL of borate buffer (made to pH 10.4). Fluorescence from the oxidized product was read from the excitation and emission scans at 310 and 414 nm wavelengths using a model LS-5B Perkin Elmer fluorometer (Beaconsfield, UK).

A fresh standard curve was performed each day using 3% H_2O_2 (Sigma) stock solution diluted in distilled water.

Reproducibility of assay

Five normal subjects performed the procedure on two different days (1 week apart) and the H_2O_2 assay was performed on these samples in the same fashion as in the study.

Statistical analyses

Results between the two groups were compared using the Mann–Whitney rank sum test. Where normality was achieved, groups were compared using the Student t-test and correlation between two variables measured by the Pearson product moment correlation test. Reproducibility was assessed using the Bland-Altman method. Statistical significance was assumed at p<0.05. Power analysis using a standard computer statistical package (SigmaStat^{TM}) showed the sample size to be adequate to give a desired power of 0.8 with alpha of 0.5 for the performed tests.

Results

Validation of assay

Salivary amylase. In all 15 samples tested, no salivary amylase was detected suggesting no contamination of breath condensate with saliva. Spiking of two samples with <0.5 mL of saliva showed levels >20,000 IU of salivary amylase in both.

Nasal air communication. Both subjects who inhaled to TLC while the nose was flushed with 100% helium had detectable helium in their expired air within the first 15 s (2.8 and 1.5%, respectively). However, exhaled air
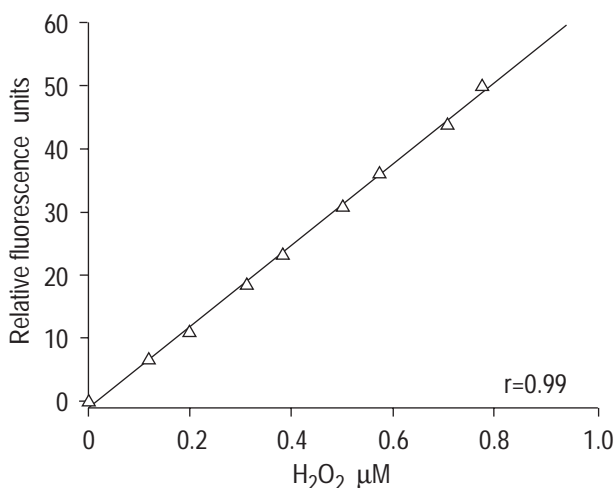
Fig. 1. – A typical calibration curve obtained with known amounts of hydrogen peroxide.

tested when helium was delivered to the nose after the start of exhalation showed no helium in the expirate. These data indicate that the method of exhaling against the resistance of the tubing is sufficient to exclude contamination of the expirate by nasal air, presumably by elevating the soft palate and sealing off the posterior nasopharynx.

Hydrogen peroxide assay. The assay was able to detect levels as low as 0.1 µM and a standard curve from 0–1 µM consistently showed a linear relationship with r=0.99 (fig. 1).

Reproducibility. A Bland-Altman test showed a mean difference between 2 days of 0.023 µM with a 95% confidence interval of -0.04–0.08 µM.

Hydrogen peroxide levels in breath condensate

The median level of H_2O_2 concentration in breath condensate of CF patients was lower than that in normal subjects (0.064 *versus* 0.090 µM) but this did not reach statistical significance (p=0.198, Mann–Whitney rank sum test). There was a larger range within the normal group (fig. 2). Within the CF group, there was no correlation
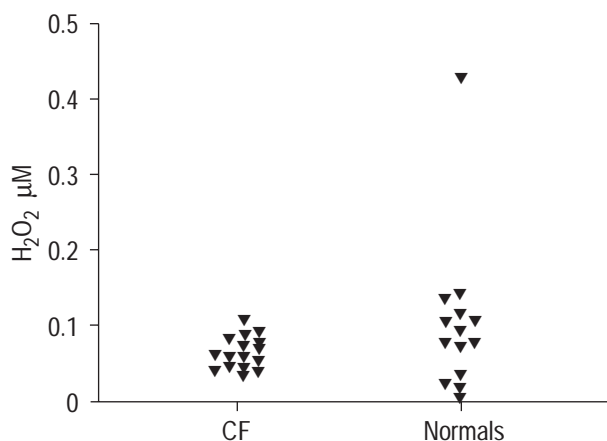

Fig. 2. – Breath condensate hydrogen peroxide in patients with cystic fibrosis (CF; n=16) and normal (n=14) subjects. There was no significant difference between the two groups (median 0.064 *versus* 0.089 µM, p=0.2).

between H_2O_2 level and lung function. The mean level of circulating leukocytes in the patient group was 12.34×10^9 cells·L^{-1} (SD 4.17×10^9 cells·L^{-1}) and that of neutrophils was 9.6×109 cells·L^{-1} (SD 4.1×10^9 cells·L^{-1}). There was a modest, but insignificant, positive correlation between H_2O_2 and circulating leukocytes and neutrophils (r=0.42, p=0.09 and r=0.44, p=0.08, respectively; Pearson product moment correlation test). Further analysis between CF patients on inhaled steroids and those not on inhaled steroids, showed no difference in mean levels between the two groups (patients on inhaled steroid (n=8) 0.066 µM and not on inhaled steroids (n=8) 0.068 µM; Student's t-test).

Discussion

We have previously shown that activated human macrophages release a large amount of H_2O_2 extracellularly [14]. Even larger amounts of H_2O_2 are released from neutrophils [15] and H_2O_2 has been measured from apical surfaces of tracheal epithelial cells, monocytes, rat alveolar type II cells and eosinophils [16, 17]. Various methods exist for measurement of H_2O_2, primarily making use of the principle of the formation of a fluorescing compound with oxidation of a stable substrate by H_2O_2. In this study, PHAA was used since it has been found that this yielded greater sensitivity and could discriminate reliably down to 0.1 µM. The standard curve was highly reproducible, with a correlation coefficient of 0.99 in five consecutive measurements (data not shown). Measurements of H_2O_2 in breath condensate, however, was more variable than those with dilutions of stock H_2O_2 in distilled water, and therefore storage, even with immediate freezing of the sample in liquid nitrogen, before or after addition of the substrate produced degradation of H_2O_2 (data not shown). Thus, all samples were measured fresh and with minimal delay. It is possible that breath condensate contains other volatile substances including glutathione or other antioxidants which may contribute to degradation of H_2O_2 with time. The levels that were observed in these CF patients (median of 0.064 µM, range 0.04–0.11 µM) were below the values reported previously by other groups working in stable asthmatic children (mean of 0.54 µM and median of 0.6 µM from two different groups) [8, 18] and stable COPD [7] (range, 0.12–0.55 µM). All the reported ranges for normal subjects: 0–0.8, 0–0.1 and 0.08–0.23 µM were similar to the levels found in this study of 0.008–0.15 µM. These values from various groups also highlight the small amount of H_2O_2 that is detectable in the breath and the need for a sensitive and robust assay.

In experimental animals, lung injury caused by complement activation can be attenuated by superoxide dismutase, catalase and desferrioxamine, antioxidants which scavenge the free radicals released by inflammatory cells [19, 20]. In ARDS, where neutrophil accumulation and stimulation is thought to amplify lung damage, glutathione (a major antioxidant in the airway epithelial fluid lining) levels have been found to be decreased [21] and H_2O_2 in the exhaled air of intubated patients, elevated [9, 10]. Thus, in CF, where neutrophilic inflammation predominates and alveolar macrophages are thought to be constantly stimulated by bacterial colonization, it was expected that H_2O_2 generation would be high and that this might be

detected in expired breath. In this study, no elevation of H_2O_2 was found in very inflamed airways.

The findings of this study were, therefore, surprising. There are several possible explanations for this. H_2O_2 may be generated, but not detected, due to rapid reaction with other reactive species, *e.g.* superoxide. Alternatively, H_2O_2 may be prevented from diffusing into the airway lumen by the large amount of viscous secretions within the airways. This is supported by a small range of H_2O_2 levels within this CF group, suggesting that, whether the levels were high or low, only a certain amount was able to escape into the lumen. In previous studies it has also been found that NO, known to be released in large quantities by neutrophils was not elevated in CF patients [22–24]. It is possible that both these reactive molecules have reacted close to their site of production, possibly encouraged by pooling of secretions and increased viscosity in the epithelial lining fluid. A third explanation may be that an increase in antioxidants like catalase or glutathione, or myeloperoxidase may be present in the CF airways environment. Myeloperoxidase generates hypohalous acids in the presence of H_2O_2 and halides, thus effectively removing H_2O_2. Both catalase and myeloperoxidase are positively charged [26], while the airway secretions are negatively charged (due to excess deoxyribonucleic acid (DNA) from degraded inflammatory cells) and thus may be trapped for a longer time in this environment. There are indirect data showing that airway secretions in CF may be "protective". *In vitro* studies have shown increased levels of free IL-8, a peptide with affinity for the anionic DNA, when recombinant DNase is added to sputum *in vitro* [25].

In conclusion, expired H_2O_2 is detectable in breath condensate down to the levels of 0.1 µM but despite a sensitive and robust assay, H_2O_2 is not elevated in cystic fibrosis breath condensate. It is thus not a good marker of airways inflammation in these patients. Whether this is due to an inherent increase in scavenging, or a physical barrier to detection was not elucidated from this study and further investigations may be of interest to explore the contribution of oxidants like H_2O_2 to lung injury of cystic fibrosis patients and the possible protective properties of airway secretions in these patients.

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