Expired hydrogen peroxide in breath condensate of cystic fibrosis patients

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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-$\beta$ 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 bronchoalveolar 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 ($H_{2}O_{2}$), released during oxidative bursts associated with sequestration of invading organisms. $H_{2}O_{2}$ is also generated through nonenzymatic and enzymatic dismutation of superoxide. Owing to the lability of superoxides, direct detection is difficult [5], but $H_{2}O_{2}$ 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 $H_{2}O_{2}$. With this method, $H_{2}O_{2}$ 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 $H_{2}O_{2}$ would be elevated in patients with CF due to the increased level of neutrophilic inflammation and activated macrophages and that $H_{2}O_{2}$ in the condensed breath may be used to follow 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.

**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
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 400–2500 mcg beclomethasone (dipropionate) and were on inhaled steroids 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 reflectometric 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 H₂O₂ was quantified using a fluorometric assay based on the formation of an intermediate compound (compound I) from H₂O₂ 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 type II horseradish 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% H₂O₂ (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 H₂O₂ 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 assessed at p<0.05. Power analysis using a standard computer statistical package (SigmaStatTM) 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

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 means±SD. M: male; F: female; FEV₁: forced expiratory volume in one second.
tested when helium was delivered to the nose after the start of exhalation showed no helium in the expire. These data indicate that the method of exhaling against the resistance of the tubing is sufficient to exclude contamination of the expire 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 H₂O₂ 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=0.198, Mann-Whitney rank sum test). There was a larger range within the normal group (fig. 2). Within the CF group, there was no correlation between H₂O₂ level and lung function. The mean level of circulating leukocytes in the patient group was 12.34×10⁶ cells·L⁻¹ (sd 4.17×10⁶ cells·L⁻¹) and that of neutrophils was 9.6×10⁹ cells·L⁻¹ (sd 4.1×10⁹ cells·L⁻¹). There was a modest, but insignificant, positive correlation between H₂O₂ 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 H₂O₂ extracellularly [14]. Even larger amounts of H₂O₂ are released from neutrophils [15] and H₂O₂ 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 H₂O₂, primarily making use of the principle of the formation of a fluorescing compound with oxidation of a stable substrate by H₂O₂. 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 H₂O₂ in breath condensate, however, was more variable than those with dilutions of stock H₂O₂ 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 H₂O₂ (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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ generation would be high and that this might be
detected in expired breath. In this study, no elevation of H$_2$O$_2$ was found in very inflamed airways.

The findings of this study were, therefore, surprising. There are several possible explanations for this. H$_2$O$_2$ may be generated, but not detected, due to rapid reaction with other reactive species, e.g. superoxide. Alternatively, H$_2$O$_2$ 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$_2$O$_2$ levels within this CF group, suggesting that, while 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$_2$O$_2$ and halides, thus effectively removing H$_2$O$_2$. 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$_2$O$_2$ is detectable in breath condensate down to the levels of 0.1 μM but despite a sensitive and robust assay, H$_2$O$_2$ 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$_2$O$_2$ to lung injury of cystic fibrosis patients and the possible protective properties of airway secretions in these patients.

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