Cyanide levels found in infected cystic fibrosis sputum inhibit airway ciliary function

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ABSTRACT   We have previously reported cyanide at concentrations of up to 150 μM in the sputum of cystic fibrosis patients infected with *Pseudomonas aeruginosa* and a negative correlation with lung function. Our aim was to investigate possible mechanisms for this association, focusing on the effect of pathophysiologically relevant cyanide levels on human respiratory cell function.

Ciliary beat frequency measurements were performed on nasal brushings and nasal air–liquid interface (ALI) cultures obtained from healthy volunteers and cystic fibrosis patients.

Potassium cyanide decreased ciliary beat frequency in healthy nasal brushings (n=6) after 60 min (150 μM: 47% fall, $p<0.0012$; 75 μM: 32% fall, $p<0.0001$). Samples from cystic fibrosis patients (n=3) showed similar results (150 μM: 55% fall, $p=0.001$). Ciliary beat frequency inhibition was not due to loss of cell viability and was reversible. The inhibitory mechanism was independent of ATP levels. KCN also significantly inhibited ciliary beat frequency in ALI cultures, albeit to a lesser extent. Ciliary beat frequency measurements on ALI cultures treated with culture supernatants from *P. aeruginosa* mutants defective in virulence factor production implicated cyanide as a key component inhibiting the ciliary beat frequency.

If cyanide production similarly impairs mucocilliary clearance *in vivo*, it could explain the link with increased disease severity observed in cystic fibrosis patients with detectable cyanide in their airway.

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Introduction

Chronic respiratory infections are currently the most lethal manifestation of cystic fibrosis, a common autosomal recessive genetic disorder caused by mutations in the ABC transporter-class chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR) [1, 2]. Impaired CFTR function deregulates normal water and ion transport and leads to a dehydrated airway surface, the production of profuse amounts of thick, viscous secretions and impaired mucociliary clearance. This leads inevitably to mucus stasis and airway obstruction that facilitates colonisation with a variety of pathogenic bacteria, the nature of which changes with age [3–5]. The lung damage that follows appears to be largely due to the complex interactions between bacterial products and a heightened inflammatory host response. A cycle of infection and consequent airway damage is established that eventually leads to respiratory failure and premature death [5]. *Pseudomonas aeruginosa* is the single most important bacterial pathogen in the cystic fibrosis airway, where it is able to establish a foothold and persist for decades [4, 6].

P. aeruginosa is one of a limited number of bacterial species that can synthesise hydrogen cyanide, a potent inhibitor of cellular respiration [7–10]. It shares this trait with the *Burkholderia cenocepacia* complex (*Bcc*), a group of clinically important lung pathogens, and it has been hypothesised that HCN production constitutes a successful infection strategy [9, 11]. HCN contributes to killing in *Caenorhabditis elegans* and *Arabidopsis thaliana* infection models and cyanogenesis is a crucial factor in the successful infection of *Drosophila melanogaster* by *P. aeruginosa* [11–13]. *In vivo* imaging, using a cyanide fluorescent chemosensor, has allowed direct observation of cyanide in the lungs of mice infected with cyanogenic *P. aeruginosa* or *Bcc* strains in an agar–bead chronic infection model [14].

We and others have previously reported HCN in the sputa of cystic fibrosis patients infected with *P. aeruginosa*, at mean concentrations of between 32–72 µM and a maximum of around 150 µM [9, 15]. HCN levels appear to be independent of bacterial load in sputum, suggesting that HCN production is strain-specific and/or a consequence of phenotypic shifts in the bacteria colonising the lung [11]. The former possibility is supported by the finding of varying degrees of HCN production amongst clinical isolates of *P. aeruginosa* when grown in culture [13, 14, 16, 17]. Furthermore, HCN production is affected by the mucoid switch that *P. aeruginosa* commonly undergoes during chronic infection and is associated with a worsened prognosis [18].

The clinical significance of cyanogenesis and its consequences for the host remain unclear [19, 20]. HCN is volatile and its usefulness as a surrogate marker for the diagnosis of *P. aeruginosa* infection in children who cannot expectorate sputum is currently being investigated [21–23].

Our previous work found that the presence of cyanide in sputum correlated negatively with lung function, with mean forced expiratory volume in 1 s (% predicted) of $26.8 \pm 3.8\%$ for patients with HCN in their sputum *versus* $46.0 \pm 6.7\%$ for those without [9]. This raises the question of whether HCN concentrations present in sputum directly impair lung function and contribute to long-term damage during infection.

Here, we have investigated the direct effect of short term exposure to pathophysiologically relevant concentrations of cyanide on human respiratory function, specifically ciliary beating. Ciliary beat frequency (CBF) measurements are a practical and accurate method of assessing the physiological status of respiratory cells and the effect of toxic compounds on their function [24]. In addition, respiratory CBF represents a clinically relevant feature, as ciliary activity is an essential component of mucociliary clearance and plays a pivotal role in preventing the development of cystic fibrosis lung disease [2, 5].

Materials and methods

Human airway epithelium collection

Strips of healthy human nasal epithelium were obtained from the inferior turbinate of volunteers using a cytology brush [25]. Cystic fibrosis samples were obtained from children undergoing clinically indicated bronchoscopy under general anaesthesia or from adults attending outpatient clinics. Brushings were dispersed by agitation into a universal tube containing 3 mL medium 199 with Earle's salts and HEPES buffer (40 mM, pH 7.4) (Invitrogen, Paisley, UK). Samples were stored at 4°C and used within 24 h of collection. The sample collection was approved by Hounslow and Hillingdon research ethics committee under project 07/H0709/73. All subjects (or parents of children) provided informed consent.

Air–liquid interface culture

Nasal brushings were cultured from a protocol adapted from a previously described method [26]. They were obtained from patients with cystic fibrosis or from patients being investigated for primary ciliary dyskinesia, but with normal results of investigations, who did not have a mucociliary clearance defect and are referred to as "non-cystic fibrosis controls". Experiments were conducted 2–6 weeks after cilia were first observed.

Harvesting bacterial culture supernatants

The *P. aeruginosa* strain PA14 and isogenic $\Delta hcnA$ and $\Delta phzS$ mutants were cultured in Luria Bertani broth (LB) (10 g·L^{-1} peptone, 5 g·L^{-1} NaCl, 5 g·L^{-1} yeast extract). Cultures were grown aerobically at 37°C in 50 mL of culture medium in a 250 mL Erlenmeyer flask with shaking at 200 rpm. Overnight cultures were adjusted to an OD$_{600}$ of 1 with LB and culture supernatants harvested by sedimenting the bacterial cells by centrifugation at $4000 \times g$ for 15 min. The supernatant fraction was then removed and filter-sterilised using a 0.2 µm pore (Pall Life Sciences, Port Washington, NY, USA).

Measurement of ciliary beat frequency

CBF was measured by an observer blinded to the experimental condition using light microscopy and high-speed video recordings as previously described [27], using an Axio observer D1 inverted microscope (Zeiss, Jena, Germany), DC60 warm stage (Linkam Scientific Instruments, Guildford, UK) and a high speed video camera (Troubleshooter; Fastcam Imaging, San Diego, CA, USA). All reagents were pre-heated to 37°C.

Following treatment with potassium cyanide, pyocyanin (PYO), KCl or 40 mM HEPES buffer, 150 µL aliquots of nasal epithelium were pipetted onto a CoverWell (Electron Microscopy Sciences, Hatfield, PA, USA) and mounted onto a slide for analysis. At each time point in the study, CBF was calculated as the mean value of six ciliated strips. Only intact ciliated strips were studied.

In order to assess reversibility of CBF inhibition, nasal strips were washed twice in PBS by centrifugation at $1500 \times g$ for 10 min and resuspended in pre-warmed PBS. CBF was measured before washing and at 30 min and 90 min after washing.

For CBF measurements of air–liquid interface (ALI) cultures, one ALI insert was used per experimental condition tested. Each insert was washed twice with 200 µL warm PBS to remove mucus then placed in a fresh 12-well culture plate for CBF analysis. The basolateral side was in cell culture media (BEGM+DMEM) (Lonza, Basel, Switzerland). 150 µL of pre-warmed PBS was added apically and baseline CBF measured for 15 min. PBS was then replaced with 150 µL of pre-warmed KCN (75 µM), PYO (75 µM), KCN/PYO (75 µM) or HEPES (40 mM).

For bacterial culture supernatant experiments, PBS was removed and replaced with 150 µL of *P. aeruginosa* culture supernatant.

CBF was calculated as the mean value of measurements made on six different areas from the ALI culture. The same six areas were used at each subsequent time point. Comparison experiments were performed on inserts from the same individual grown at the same time. Replicates of experiments were made on ALI cultures from different individuals.

Viability measurement

Cell viability was determined using trypan blue staining [28]. Following treatment, 50 µL aliquots of nasal brushings were incubated for 60 min at 37°C. 50 µL trypan blue was then added and cells were disaggregated by gentle pipetting. Cell viability was assessed by direct cell counting of the number of stained and unstained cells in a haemocytometer.

ATP measurements

Following 1-h treatment with HEPES, KCN or pyocyanin, cellular ATP content was measured using the CellTiter Glo (Promega, Madison, WI, USA) luminescent cell viability assay according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SEM. Results were submitted to analysis of variance (ANOVA) followed *post hoc* by the Tukey test using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Due to variation in baseline CBF between ALI cultures originating from the same patient, data from ALI experiments are expressed as percentage of baseline. The null hypothesis was rejected at $p < 0.05$.

Results

Cyanide decreases ciliary beat frequency of nasal brushings

To test the effect of cyanide on epithelial cell function, we monitored the CBF of human nasal brushings, obtained from healthy volunteers, following exposure to physiologically relevant levels of KCN (150 µM and 75 µM; fig. 1). When compared with HEPES and KCl controls, 150 µM KCN caused a significant decrease in CBF after 60 min (47% fall, $p < 0.001$, n=6; fig. 1a). This effect was apparent within the first 15 min and maximal by 30 min. The inhibitory effect of KCN on CBF was also seen in nasal brushings from cystic fibrosis patients (55% fall, $p < 0.001$, n=3; fig. 1b). The inhibitory effect of KCN on CBF was
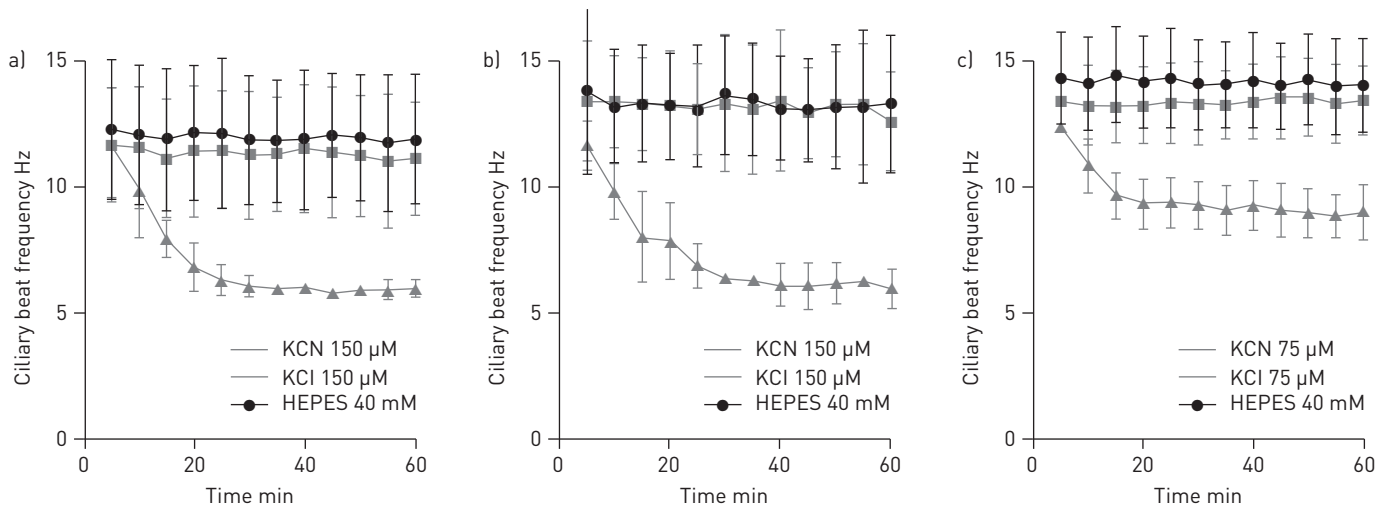
FIGURE 1 Effect of cyanide (KCN) on ciliary beat frequency (CBF) of human nasal brushings. a) Healthy volunteers (n=6). Baseline CBF 11.4 Hz, 150 µM KCN CBF 5.9 Hz; 47% fall after 60 min exposure (p<0.001). b) Cystic fibrosis patients (n=3). Baseline CBF 13.3 Hz, 150 µM KCN CBF 5.9 Hz; 55% fall after 60 min exposure (p<0.001). c) Healthy volunteers (n=6). Baseline CBF 13.4 Hz, 75 µM KCN CBF 9.0 Hz; 32% fall after 60 min exposure (p<0.0001).

concentration dependent, with 75 µM causing a lesser but still significant decrease (32% fall, p<0.0001, n=6; fig. 1c).

Cyanide-mediated inhibition of ciliary beat frequency is reversible

The fall in CBF was not due to a loss of cell viability based on trypan blue staining (nonsignificant compared to KCl; fig. 2a). Furthermore, following inhibition of CBF with 150 µM KCN, CBF recovered to baseline levels 30 mins after cyanide was removed by washing (fig. 2b) (30 min 6.5 Hz, 54% fall, p<0.0001; 60 min 13.8 Hz; NS), and CBF was still normal after 120 min (13.85 Hz NS). These data indicate that KCN inhibition of CBF over the time period studied is transient and reversible.

Combined effect of cyanide and pyocyanin on ciliary beat frequency of nasal brushings

Like cyanide, the *P. aeruginosa* virulence factor pyocyanin is detectable in sputum at concentrations of 75–100 µM [29]. To evaluate the implications of the presence of both PYO and cyanide in the lung, we tested their combined effect on CBF. Nasal brushings from healthy volunteers were exposed to KCN, PYO or KCN+PYO for 60 min (fig. 3). In accordance with other work, 75 µM pyocyanin decreased CBF (27%
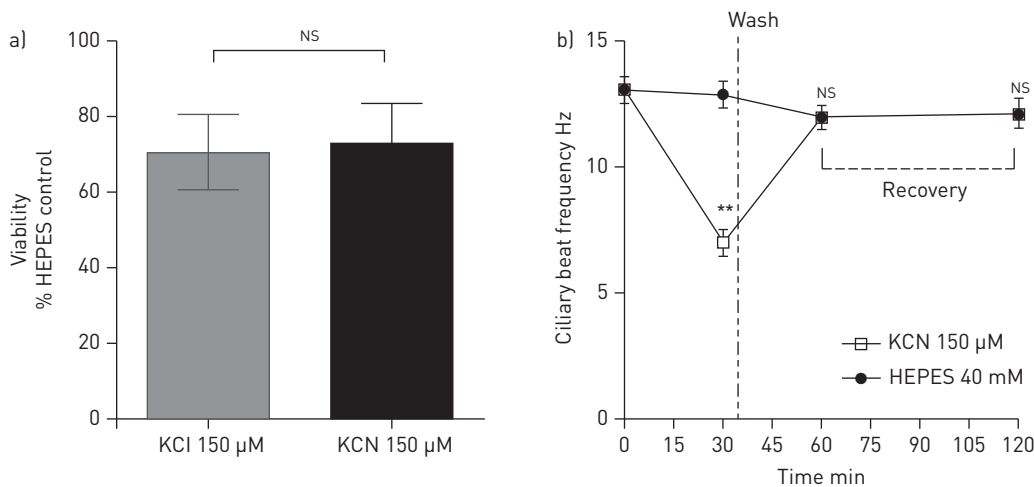

FIGURE 2 Reversibility of ciliary beat frequency (CBF) inhibition. a) KCN has no effect on viability after 60 min exposure (nonsignificant). b) KCN inhibition of CBF is reversible. Nasal brushings (n=6) were exposed to 150 µM KCN and after the 30 min CBF measurement they were washed to remove KCN. Baseline CBF 14 Hz, and after 30 min exposure to 150 µM KCN CBF 6.5 Hz; 54% fall (p<0.0001). At 60 min CBF 13.8 Hz (nonsignificant compared to HEPES control), and at 120 min CBF 13.9 Hz (nonsignificant compared to HEPES control).
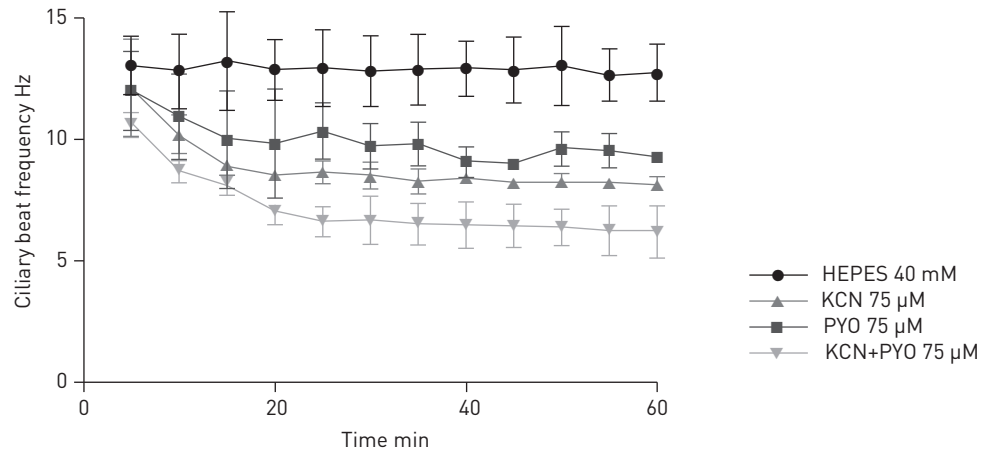
FIGURE 3 Effect of cyanide (KCN) and pyocyanin (PYO) on ciliary beat frequency (CBF) of nasal brushings. Healthy volunteers (n=3). Baseline CBF 12.7 Hz, 75 µM PYO CBF 9.3 Hz; 27% fall after 60 min (p<0.001); 75 µM KCN CBF 8.1 Hz; 42% fall after 60 min (p<0.0001); PYO+KCN CBF 6.2 Hz; 51% fall after 60 min (p<0.005).

fall compared to KCl control; p<0.001), but this effect was less marked than with physiologically relevant concentrations of cyanide, as treatment with 75 µM KCN caused a 42% fall in CBF compared to the KCl control (p<0.0001). Combined treatment with KCN+PYO caused a greater decrease than treatment with PYO alone (51% fall; p<0.005).

Combined effect of cyanide and pyocyanin on ciliary beat frequency of air–liquid interface cultures

We investigated the effect of cyanide and pyocyanin in the ALI model. CBF of ALIs was measured following treatment with KCN, PYO or KCN+PYO (fig. 4a). After 60 min, the compounds tested significantly inhibited CBF but to a lesser extent than observed in the nasal brushing model. 75 µM KCN caused a 13% fall in CBF (p<0.0001; n=4), while 75 µM PYO caused a 5% decrease (p<0.05; n=4). Combined treatment with 75 µM PYO+KCN resulted in an 18% fall in CBF (p<0.0001; n=4). This decrease was significantly greater than that observed following treatment with pyocyanin alone (p=0.0015), but was not significantly greater than treatment with KCN alone. We also confirmed these observations in ALIs cultured from cystic fibrosis patients (75 µM KCN 20% fall, p<0.0001; 75 µM PYO 10% fall, p=0.001; 75 µM PYO+KCN 22% fall, p<0.0001; n=3; fig. 4b). These results suggest that some of the inhibitory effects of pyocyanin observed in the nasal brushing model are less marked in ALI cultures and that cyanide appears to be responsible for most of the inhibitory effects on CBF observed in the ALI model.

Effect of cyanide and pyocyanin on ATP levels of nasal brushings

Given that cyanide is a mitochondrial cytochrome c oxidase inhibitor, we determined whether the mechanism of CBF reduction was *via* the depletion of ATP levels. ATP levels in nasal brushings were measured after 60 min exposure to KCN, PYO or KCN+PYO (fig 5). Concordant with previous findings, pyocyanin caused a marked decrease in ATP levels (75 µM PYO 70% fall in ATP; p<0.0001; n=5) [30]. However, 75 µM KCN had no significant effect on ATP levels. Combined treatment with 75 µM KCN+PYO did not cause a significantly greater decrease in ATP levels than treatment with PYO alone. These findings demonstrate that KCN does not lead to a decrease in ATP levels and suggest that KCN and pyocyanin have differing mechanisms of CBF inhibition.

Effect of P. aeruginosa culture supernatants on ciliary beat frequency of nasal brushings

The production of HCN and pyocyanin is coordinately regulated in *P. aeruginosa* and both components accumulate extracellularly in sputum. Therefore, we tested the effect on CBF of supernatant fractions from cultures of a *P. aeruginosa* wild-type strain (PA14), a cyanide-deficient mutant (ΔhcnA) and a pyocyanin-deficient mutant (ΔphzS) (fig. 6). As measured with a cyanide selective electrode [31], the culture supernatants of PA14 and ΔphzS strains contained 73 µM and 75 µM cyanide in solution, respectively, whereas no detectable cyanide was present in the ΔhcnA strain. The culture supernatant from PA14 caused a significant decrease in CBF after 60 min (23% fall; p<0.001). No inhibition was observed with the culture supernatant from ΔhcnA (p=NS), whereas supernatant from the ΔphzS strain inhibited CBF at levels similar to wild-type (19% fall; p<0.001). These results confirm an important role of cyanide in the cilio-inhibitory effect of *P. aeruginosa* culture supernatant and suggest that, at least under the growth conditions used here, cyanide is the main extracellular component produced by *P. aeruginosa* that affects CBF.
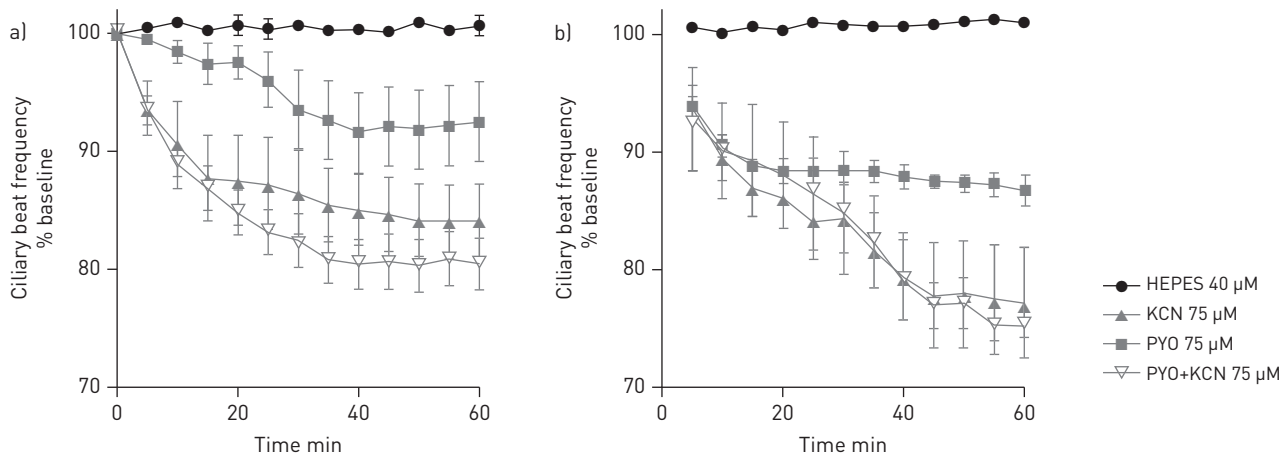
FIGURE 4 Effect of pyocyanin (PYO) and cyanide (KCN) on ciliary beat frequency (CBF) of air–liquid interface (ALI) cultures. a) Non-cystic fibrosis controls (n=4). Baseline CBF 11.7 Hz; 75 µM KCN 13% fall in CBF compared to control after 60 min (p<0.0001); 75 µM PYO 5% fall in CBF compared to control after 60 min (p<0.05); 75 µM PYO+KCN 18% fall in CBF compared to control after 60 min (p<0.0001). b) Cystic fibrosis patients (n=3). Baseline CBF 12.9 Hz; 75 µM KCN 20% fall in CBF compared to control after 60 min (p<0.0001); 75 µM PYO 10% fall in CBF compared to control after 60 min (p=0.001); 75 µM PYO+KCN 22% fall in CBF compared to control after 60 min (p<0.0001).

Discussion

We previously found that cyanide was detectable in the lungs of cystic fibrosis patients infected with *P. aeruginosa*, where its presence was negatively correlated with lung function [9]. However, as cyanide production is regulated by quorum sensing and oxygen concentration (*via* the transcriptional regulator ANR [8]) the loss of lung function associated with its presence in sputum might have been a consequence of other quorum sensing or ANR-regulated changes, and not due directly to cyanide itself. Here, we tested the hypothesis that cyanide, at concentrations similar to those found in sputum, could be of clinical relevance and contribute to disease severity through a direct effect on lung tissue and in particular epithelial cell function. We focused on the effect of short-term cyanide exposure on ciliary beating, an essential component of mucociliary clearance in the lung, and used two human model systems: strips of ciliated human nasal epithelium and nasal ALI cultures.

The kinetics of cyanide inhibition of CBF we report here are broadly concordant with those observed in studies looking at the effects of cigarette smoke condensate on non-human ciliated models, such as freshwater mussel gills and chicken trachea [32–34]. As with our study, the time scales investigated were short (1–2 h). As such, future experiments will be needed to determine the effect of longer term exposure of epithelial cells to cyanide to better relate these to chronic cyanide exposure in the cystic fibrosis lung.

Within the time frame studied, sub-lethal cyanide levels caused a concentration-dependent and reversible decrease in CBF. Interestingly, CBF slowed to a minimum level beyond which no further decrease was observed; further, the effects are not linked to a loss of cellular viability. *P. aeruginosa* releases a range of extracellular virulence factors that are thought to affect lung function of cystic fibrosis patients [6], that
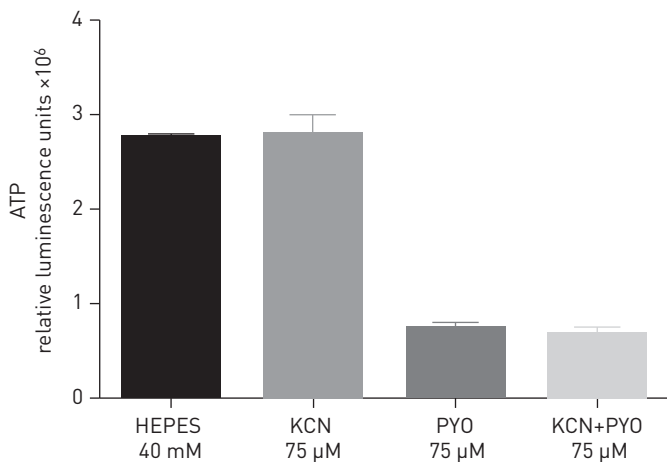

FIGURE 5 Effect of pyocyanin (PYO) and cyanide (KCN) on ATP levels. Nasal brushings from healthy volunteers (n=5). 75 µM PYO 70% fall in ATP levels compared to control (p<0.0001); 75 µM KCN no significant change in ATP levels.
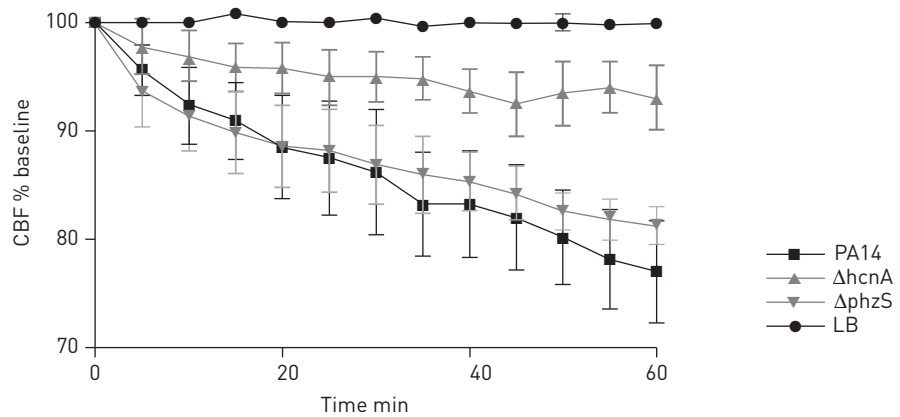
FIGURE 6 Effect of *Pseudomonas aeruginosa* culture supernatants on ciliary beat frequency (CBF) of air–liquid interface (ALI) cultures. Non-cystic fibrosis controls (n=3). Baseline CBF was 13.8 Hz. Culture supernatant or Luria Bertani broth (LB) (control) was added to ALI cultures and CBF measured after 60 min. Addition of culture supernatant of PA14 led to 23% fall in CBF (p<0.001) after 60 mins; culture supernatant from *ΔhcnA* mutant led to no significant inhibition (P=0.33), and that from a *ΔphzS* mutant to a 19% fall in CBF (p<0.001).

include exotoxin A, elastase, LasA protease and pyocyanin. Pyocyanin, at a concentration typical of those found in sputum (75 μM), inhibited CBF to a lesser extent than cyanide and this effect was smaller in ALI cultures.

The data presented here suggest that cyanide plays a major role in the inhibition of CBF *in vitro*, a conclusion supported by experiments using supernatants from *P. aeruginosa* cultures. Culture supernatants from a pyocyanin deficient mutant were able to inhibit CBF to a similar extent as the wild-type, while low levels of inhibition were observed with a cyanide-deficient mutant.

In accordance with a previous report, pyocyanin inhibition of CBF was associated with depletion of ATP [30]. The inhibitory effect of cyanide, by contrast, was not linked to ATP depletion, suggesting that the detrimental effect of cyanide in this context may not be as a consequence of inhibition of mitochondrial respiration. One possible explanation is that mitochondria possess a rhodanese and a mercaptopyruvate sulfurtransferase that can detoxify cyanide [35, 36], and that they have sufficient activity to cope with the external cyanide concentrations present in the *P. aeruginosa*-infected lung.

Reversal of CBF inhibition could be beneficial to patients. Work on isolated sheep trachea has shown that a 16% increase in CBF can lead to a 56% increase in mucociliary clearance [37]. While basal CBF is not affected in cystic fibrosis, abnormal mucociliary clearance is thought to be the key to the development of lung disease in cystic fibrosis. However, clinical studies are not consistent in demonstrating a cystic fibrosis -related defect in the mucociliary clearance and recent data have implicated the need for a secondary effect, for example viral infection to permanently damage the mucociliary clearance. In the largest clinical study to date it was found that cystic fibrosis patients had mucociliary clearance approximately 50% of the normal rate and data suggest that there is no primary mucociliary clearance defect in cystic fibrosis, but rather a gradual decrease with increased lung disease [5].

At present, several cystic fibrosis therapeutics have been developed to stimulate mucociliary clearance, including hypertonic saline, mannitol, amiloride and UTP. Our findings suggest that diluting cyanide concentrations with these therapies could contribute to their beneficial effect. An alternative strategy could be to detoxify cyanide directly, for example through complex formation with hydroxycobalamin (a vitamin B12 precursor), which is already used to counter cyanide toxicity in smokers and victims of fires [38, 39].

Since *P. aeruginosa* is an important pathogen in other lung diseases, including acute exacerbations of chronic obstructive pulmonary disease and in non-cystic fibrosis bronchiectasis, it is plausible that cyanide production could affect CBF and, therefore, mucociliary clearance in these diseases. Indeed, cyanide has been detected in sputum of non-cystic fibrosis bronchiectasis patients infected with *P. aeruginosa* at similar levels to those present in cystic fibrosis patients [9].

In conclusion, we have shown that levels of cyanide similar to those found in *P. aeruginosa*-infected sputum have a significant effect on respiratory cells. This finding could explain the decrease in lung function associated with the presence of cyanide in sputum and supports the use of therapeutic interventions that stimulate mucociliary clearance, or dilute out or detoxify cyanide.

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