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Nitric oxide production by polymorphonuclear leucocytes in infected cystic fibrosis sputum consumes oxygen

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Summary

Chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis (CF) patients is characterized by persisting mucoid biofilms in hypoxic endobronchial mucus. These biofilms are surrounded by numerous polymorphonuclear leucocytes (PMNs), which consume a major part of present molecular oxygen (O_2) due to production of superoxide (O_2^-). In this study, we show that the PMNs also consume O_2 for production of nitric oxide (NO) by the nitric oxide synthases (NOS) in the infected endobronchial mucus. Fresh expectorated sputum samples ($n = 28$) from chronically infected CF patients ($n = 22$) were analysed by quantifying and visualizing the NO production. NO production was detected by optode measurements combined with fluorescence microscopy, flow cytometry and spectrophotometry. Inhibition of nitric oxide synthases (NOS) with N^G-monomethyl-L-arginine (L-NMMA) resulted in reduced O_2 consumption ($P < 0{\cdot}0008$, $n = 8$) and a lower fraction of cells with fluorescence from the NO-indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) ($P < 0{\cdot}002$, $n = 8$). PMNs stained with DAF-FM and the superoxide indicator hydroethidine (HE) and host cells with inducible NOS (iNOS) were identified in the sputum. In addition, the production of the stable end-products of NO in CF sputum was correlated with the concentration of PMNs; NO_3^- ($P < 0{\cdot}04$, $r = 0{\cdot}66$, $n = 10$) and NO_2^- ($P < 0{\cdot}006$, $r = 0{\cdot}78$, $n = 11$). The present study suggests that besides consumption of O_2 for production of reactive oxygen species, the PMNs in CF sputum also consume O_2 for production of NO.

Keywords: cystic fibrosis, neutrophil biology, nitric oxide, pneumonia, *Pseudomonas aeruginosa*

Introduction

Cystic fibrosis (CF) is an autosomal recessive inherited disorder characterized by accumulation of large volumes of thick, endobronchial mucus that increase the risk for severe chronic *Pseudomonas aeruginosa* infections [1]. Chronic *P. aeruginosa* lung infection is characterized by the formation of biofilm aggregates surrounded by numerous polymorphonuclear leucocytes (PMNs) in the endobronchial mucus [2]. The biofilm mode of growth protects the bacteria from antibiotics and shields against host defences such as bactericidal defence mechanisms of the activated PMNs [2]. Prolonged activation of PMNs in chronic lung infection can cause progressive lung tissue damage by the release of proteolytic enzymes and reactive oxygen species (ROS) [1,3]. However, the activity of PMNs in chronic *P. aeruginosa* lung infection in CF patients is, by far, not fully understood. We recently observed ongoing respiratory burst in PMNs of expectorated endobronchial secretions and have demonstrated that the O_2 consumption by PMNs during the respiratory burst is the major cause of O_2 depletion, leading to anoxia in the endobronchial mucus in CF patients with chronic *P. aeruginosa* lung infection [4,5]. In addition, only a minute part of the O_2 is consumed by aerobic respiration in endobronchial secretions from CF patients [5], and the production of nitrous oxide (N_2O) in CF sputum [6] indicates that *P. aeruginosa* adapts to the anoxia by employing anaerobic respiration by

denitrification to obtain energy for growth. Ongoing denitrification by *P. aeruginosa* in the infected CF lung is corroborated further by the increased expression of genes involved in denitrification in the virulent mucoid isolates [7]. Besides O_2 consumption during the respiratory burst, where the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX-2) reduces O_2 to superoxide (O_2^-) [8], activated PMNs may also consume O_2 for nitric oxide (NO) production [9] by incorporating O_2 into both NO and L-citrulline during the enzymatic oxidation of L-arginine and reduction of O_2 by nitric oxide synthases (NOS) [10–13]. Human PMNs exhibit a constitutive expression of inducible nitric oxide synthases (iNOS) [14], which may participate in the NO production in PMNs isolated from bacterial infections [10], as well as in the nitration of bacteria ingested by PMNs *in vitro* [11] and in the formation of stable nitrogen species such as nitrate (NO_3^-) and nitrite (NO_2^-) resulting from the rapid reaction of NO with other molecules, such as O_2^- [15]. Both iNOS and the phagocytic NOX-2 can be activated by bacterial and inflammatory stimuli [16], and we therefore hypothesized that PMNs, in addition to utilizing O_2 for the formation of O_2^-, also consume O_2 for the production of NO in the lungs of CF patients with chronic *P. aeruginosa* lung infection. In view of the fact that *P. aeruginosa* up-regulates genes for denitrification in response to the presence of NO and to O_2 depletion [17], the simultaneous reduction of O_2 consumption and NO production without decreasing products from the NOX-2 in stimulated human PMNs by inhibition with L-NMMA [9] motivated our use of N^G-monomethyl-L-arginine (L-NMMA) to estimate ongoing NO production and the associated O_2 consumption by PMNs in CF sputum.

Because NO is an unstable molecule with the ability to produce various end-products, including NO_3^- and NO_2^- [15,18], several methods were applied to display the NO production by PMNs in fresh expectorated sputum from CF patients with chronic *P. aeruginosa* lung infection. These methods include the demonstration of NO in the sputum samples by NOS-dependent staining of sputum cells with fluorescent indicators for NO, estimation of NOS-dependent O_2 consumption, specific staining of iNOS expression in sputum cells as well as correlating the concentration of PMNs to NO_3^- and NO_2^- concentrations in sputum.

Materials and methods

Sputum samples

As defined by the Danish Act on Research Ethics Review of Health Research Projects, Section 2, the project does not constitute a health research project and was thus initiated without approval from the Committees on Health Research Ethics in the Capital Region of Denmark. Therefore, verbal informed consent was obtained using waiver of documentation of consent. The study was carried out on 28 anonymous samples of surplus expectorated sputum collected for routine bacteriology from 22 CF patients with chronic *P. aeruginosa* infections (Table 1). Chronic *P. aeruginosa* infection was defined as the presence of *P. aeruginosa* in the lower respiratory tract in each monthly cultivation from sputum samples for > 6 months, or for a shorter time in the presence of increased antibody response to *P. aeruginosa* (more than two precipitating antibodies, normal: 0–1) [19]. The experiments were performed before elective intravenous antibiotic treatment of the CF patients for 2 weeks every 3–4 months [20,21].

Table 1. Demographic data of cystic fibrosis (CF) patients.

Characteristic	
Number (male)	22 (9)
Age (years)†	38 (23–47)
Duration of chronic infection (years)†	9 (4–19)
FEV_1 (%)†	48 (24–82)
FVC (%)†	84 (27–138)

†Values are medians (range). FEV_1 = forced expiratory volume in 1 s; FVC = forced vital capacity.

Concentration of dissolved O_2

Sputum samples were equilibrated to normoxic conditions by diluting 10 times with Krebs–Ringer buffer [130 mM NaCl, 5 mM KCl, 0·9 mM $CaCl_2$, 1·2 mM $MgCl_2$, 15 mM NaH_2PO_4 (pH = 7·4)] with 10 mM glucose equilibrated in ambient air. Dissolved O_2 in the sample was measured in a reaction chamber fixed on top of a luminescent dissolved oxygen (LDO) sensor connected to an HQ40d meter (Hach Company, Loveland, CO, USA), as described previously [5]. To separate the O_2 consumed by NOS from total consumption, sputum was pretreated with the NOS-inhibitor, 2 mM N^G-monomethyl-L-arginine (L-NMMA) (M7033; Sigma, St Louis, MO, USA) for 10 min at 37°C before recording the concentration of O_2 in the samples for 30 min.

Visualization of NO production

Sputum was stained with 20 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) (D23842; Life Technologies, Copenhagen, Denmark) and shaken for 30 min at 37°C. To verify that the NO content was related to NOS, sputum samples were pretreated with 2 mM L-NMMA for 10 min before the addition of DAF-FM. Detection of PMNs engaged in NO production in sputum was performed by staining with 5 μM DAF-FM and 5 μM hydroethidine (HE) (D-23107, Life Technologies) to identify PMNs with an ongoing production O_2^-. After incubation the samples were examined with a fluorescence

microscope (BX40; Olympus, Tokyo, Japan) equipped with a fluorescein isothiocyanate-propidium iodide (FITC-PI) filter.

Quantification of intracellular NO

DAF-FM-stained sputum was washed in Krebs–Ringer buffer and fixated in 2% paraformaldehyde (PFA) (P-6148; Sigma-Aldrich, St Louis, MO, USA) in phosphate-buffered saline (PBS), pH 7·4. Cells in the fixed sputum were stained with PI (100 μg ml^{-1}) (P-4170; Sigma-Aldrich) before flow cytometry. To avoid influence of the time-period between the fixation and flow cytometry, fixated samples were analysed 15 min after fixation.

Intracellular staining of iNOS in sputum

Sputum was fixated in 2% PFA in PBS (126101; USB Corporation, Cleveland, OH, USA). Fixed cells were permeabilized with 0·2 % Triton X-100 (45H04811; Sigma) and washed in PBS before incubation with immunoglobulin (Ig)G2a FITC-labelled anti-iNOS (BD 610331; Becton Dickinson, Albertslund, Denmark) and the FITC-labelled mouse isotype control (BD 340459; Becton Dickinson) diluted in mouse sera (X0910; Dako, Glostrup, Denmark) overnight on ice. Cells were stained with PI before flow cytometry.

PMN concentration

The concentration of PMNs was estimated as described previously [5]. Briefly, the concentration of leucocytes in the sputum was determined by adding 100 μl diluted sputum to a TrueCount tube (BD 340334; Becton Dickinson) with 400 μl fluorescence activated cell sorter (FACS) lysis solution (BD 349202; Becton Dickinson) with PI (100 μg ml^{-1}) and the samples were incubated for at least 10 min prior to flow cytometry. The frequency of PMNs was estimated by the addition of 5 μl of each of the following mouse anti-human monoclonal antibodies from BD Bioscience: phycoerythrin-labelled CD11b (555388), peridinin chlorophyll A protein-labelled CD14 (345786), FITC-labelled CD15 (555401) and allophycocyanin (APC)-labelled CD45 (555485) to 100 μl diluted sputum. The samples were incubated on ice for 30 min, washed with cold PBS and fixed in PBS with 2% PFA before they were analysed by flow cytometry. The concentration of PMNs was calculated by multiplying the concentration of leucocytes with the fraction of PMNs.

Flow cytometry

The samples were analysed using a FACSort (BD Bioscience, La Jolla, CA, USA) equipped with a 15 mV argon-ion laser tuned at 488 nm, and a red diode laser emitting at 635 nm for excitation. Light-scatter, time and exponentially amplified fluorescence parameters from at least 10 000 events were recorded in list mode. Host cells were discriminated according to their DNA content and their morphology by gating on the PI fluorescence intensity and light-scatter. Leucocytes were discriminated by gating on CD45 and the PMNs were discriminated by gating on CD11b, CD14 and CD15, as described previously [5]. The instrument was calibrated using Calibrite beads (BD Bioscience).

NO_3^- and NO_2^- quantification

The concentration of NO_3^- and NO_2^- in sputum was measured in 11 samples using the Griess colorimetric reaction (no. 780001; Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's recommendations. From each sputum sample, 100 μl were immediately diluted ×10 in PBS and stored at −20°C for later analysis. Frozen sputum samples were transferred to a 96-well microtitre plate. NO_2^- concentration was estimated by addition of the Griess reagent for 10 min, whereby NO_2^- was converted into a purple azo-compound, which was quantitated by the optical density at 540–550 nm measured with an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Scientific Multiskan EX; Thermo Fisher Scientific Inc., BioImage, Søborg, Denmark). Total NO_3^- and NO_2^- levels were estimated by a two-step analysis process: the first step converted NO_3^- to NO_2^- utilizing NO_3^- reductase. After incubation for 2 h, the next step involved the addition of the Griess reagent, whereby NO_2^- was converted into a purple azo-compound. After incubation with Griess reagent for 10 min the optical density at 540–550 nm was measured with an ELISA plate reader (Thermo Scientific Multiskan EX; Thermo Fisher Scientific Inc., BioImage). A NO_3^- standard curve was used for determination of total NO_3^- and NO_2^- concentration, while a NO_2^- standard curve was used for determination of NO_2^- alone. The concentration of NO_3^- was calculated as the difference between the NO_3^- concentration and the total NO_3^- and NO_2^- concentration.

NO-microprofile

A sputum sample was added to a well in a microtitre plate (Nunc, Roskilde, Denmark). A NO-microprofile in the sputum sample was then monitored with an amperometric microsensor (Unisense, Århus, Denmark). The microsensor was prepared and calibrated as described previously [22]. The microsensor tip with a tip diameter of 80 μm was adjusted manually to the upper surface of the sample and vertical concentration profiles were measured by using a semi-automated set-up. The microsensor was mounted on a micromanipulator (Translation Stage VT-80; Micos, Irvine, CA, USA) and connected to a picoammeter PA2000 (Unisense). Measurements were controlled by Sensortrace Pro version 2·0 (Unisense). Profile measurements were permitted by movement of the sensor in 100-μm steps through the sputum sample.

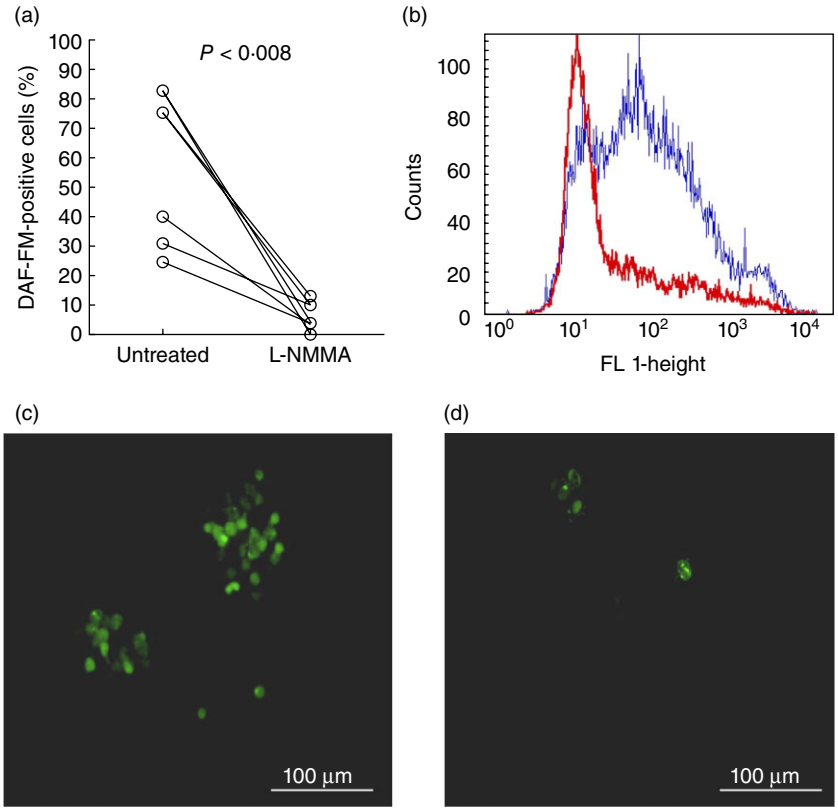

Fig. 1. Nitric oxide (NO) expression in sputum samples from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* lung infection. (a) Mean percentage of cells positive for 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) fluorescence in untreated sputum and in sputum treated with 2 mM NG-monomethyl-L-arginine (L-NMMA) ($P < 0{\cdot}002$, $n = 8$). Statistical analysis was performed by Wilcoxon's signed-rank test. (b) Representative example of intracellular DAF-FM fluorescence (FL h1- height) in untreated sputum cells (blue histogram) and L-NMMA-treated sputum cells (red histogram). (c) Visualization of NO production by fluorescence microscopy (green colour) of cells stained with (DAF-FM) in untreated sputum and (d) sputum treated with 2 mM L-NMMA to inhibit nitric oxide synthase (NOS).

Statistical methods

Statistical significance was evaluated by Wilcoxon's signed-rank test and Spearman's rank correlation test. A P-value $< 0{\cdot}05$ was considered statistically significant. The tests were performed with Prism version 4·0c (GraphPad Software, La Jolla, CA, USA).

Results

NO in host cells infected sputum from CF patients

Staining cells with the NO indicator DAF-FM, a fluorescent dye that expresses fluorescence after the intracellular reaction with an intermediate of NO formed during the spontaneous oxidation of NO to NO_2^- [23], allowed quantification of sputum host cells with NO generation. Flow cytometry showed that a median of 75% of the cells (range 25–83%) in untreated sputum were positive for NO expression (Fig. 1a).

Inhibition of NOS with L-NMMA resulted in an approximately 90% reduction of DAF-FM-positive cells (Fig. 1b), indicating that active NOS in PMNs is the major contributor to the NO formation in sputum. This was confirmed by visual inspection of untreated and L-NMMA-treated sputum samples stained with DAF-FM (Fig. 1c,d).

O_2 consumption by NOS- and iNOS-positive cells in infected sputum from CF patients

Initially, the expectorated sputum was diluted 10 times in the Krebs–Ringer buffer equilibrated in ambient air to ensure similar O_2 concentrations. Subsequently, O_2 consumption was observed in all sputum samples (Fig. 2). When sputum was treated with L-NMMA the rate of O_2 consumption decreased, which is in accordance with the O_2 consumption by NOS in activated PMNs [9]. The decreased O_2 consumption indicates that the contribution of NOS to the total O_2 consumption approximated 28% (range 9–37%) (Fig. 2).

The high fraction of cells in sputum with specific FITC-labelled anti-iNOS antibody, compared to the low fluorescence intensity of cells stained with FITC-labelled isotype control (Fig. 3), suggests that the majority of sputum cells is equipped with enzymes needed for production of NO. Because PMNs predominate the host cell population in CF sputum and CF epithelial cells have low expression of iNOS [24], this suggests that the PMNs employ iNOS for NO production in CF sputum.

NO production in PMNs in infected sputum from CF patients

Simultaneous staining with DAF-FM and HE in sputum enabled us to identify sputum PMNs that produce NO

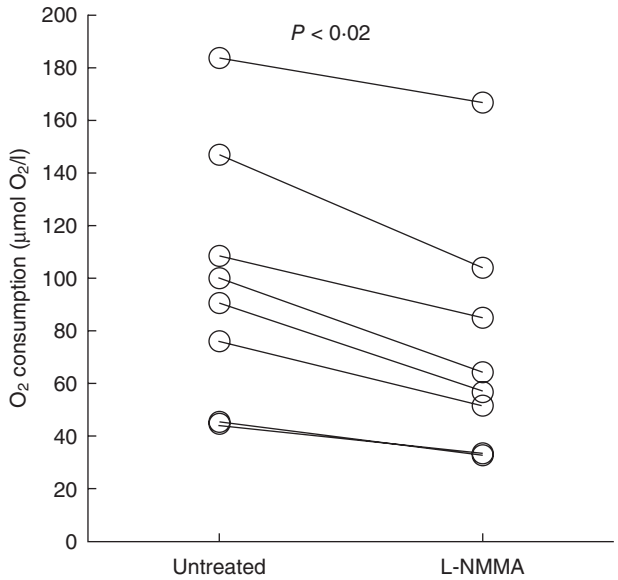

Fig. 2. Molecular oxygen (O_2) consumption by inducible nitric oxide synthase (iNOS) in sputum from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* lung infection. Decreased O_2 consumption in sputum during treatment with 2 mM NG -monomethyl-L-arginine (L-NMMA) ($P < 0{\cdot}008$, $n = 8$) compared to untreated sputum. Statistical analysis was performed by Wilcoxon's signed-rank test.

simultaneously with O_2^- due to the green fluorescence from NO reacting with DAF-FM in the cytoplasm and the red fluorescent multi-lobed nuclei resulting from staining of the DNA caused by the product from HE being oxidized by O_2^- [25] (Fig. 4). To ensure that the chemical reaction between the two probes did not cause an experimental artefact, we used flow cytometry to compare the green and red fluorescence from human PMNs stained with DAF-FM, HE and DAF-FM together with HE. By this means, the cross-interaction was calculated to account for less than 5% of the fluorescence of double-stained PMNs (data not shown). This level of cross-interaction was considered too low to interfere with the interpretation of the results.

NO_3^- and NO_2^- concentration in infected sputum from CF patients

The simultaneous formation of NO and O_2^- observed in PMNs in CF sputum (Fig. 4) is likely to cause rapid formation of peroxynitrite ($ONOO^-$) [26]. $ONOO^-$ may then disintegrate to NO_3^- and NO_2^- accumulation in sputum [18]. Thus, the concentration of PMNs was compared with the concentration of NO_3^- and NO_2^- in infected sputum, resulting in significant correlations (Fig. 5).

NO in sputum from CF patients with chronic *P. aeruginosa* lung infection

The sputum occasionally revealed discrete occurrences of NO when we injected a NO microsensor in to the sputum sample (Fig. 6). We measured a concentration of up to 4 µM of NO in the sputum. We believe that the profile demonstrates localized secretion of NO, which is rapidly lost due to the instability of NO [13,15,16].

Discussion

NO production by NOS in CF airways infected with *P. aeruginosa* has been demonstrated previously by increased levels of exhaled NO following L-arginine inhalation [27]. However, the cellular sources of NOS activity have so far not been demonstrated firmly, as iNOS expression and NO production is reduced in CF lung epithelial cells during proinflammatory stimulation [28]. In the present study we now provide substantial evidence for the presence of ongoing NO production in PMNs in freshly expectorated sputum from CF patients with chronic *P. aeruginosa* lung infection. iNOS was detected in the majority of sputum cells and iNOS may be activated in human alveolar macrophages [29], neutrophils [14,30,31] and inflammatory cells from CF airways [32]. Therefore, other cell types than PMNs may contribute to the observed NO production in sputum. The PMNs, however, constitute the major host cell population in sputum as the host cell population in CF sputum consists of leucocytes and epithelial cells, which rarely exceed 20% of the total host cell population [33], and the PMNs constitute from 96 to 99% of the leucocytes [34,35]. In addition, CF epithelial cells produce low amounts of NO [28]. Accordingly, we propose

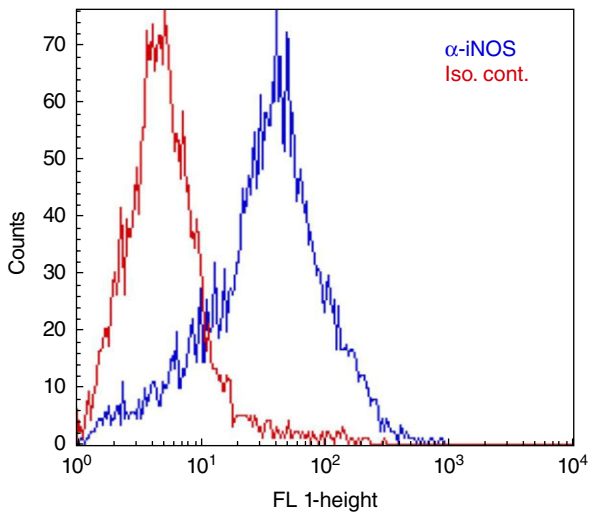

Fig. 3. Inducible nitric oxide synthase (iNOS)-positive cells in sputum from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* lung infection. Representative flow cytometric histogram of sputum cells demonstrating high fluorescence (FL 1-height) in sputum stained with specific fluorescein isothiocyanate (FITC)-labelled anti-iNOS antibody (blue histogram) and low fluorescence in sputum stained with FITC-labelled isotype control antibody (red histogram).

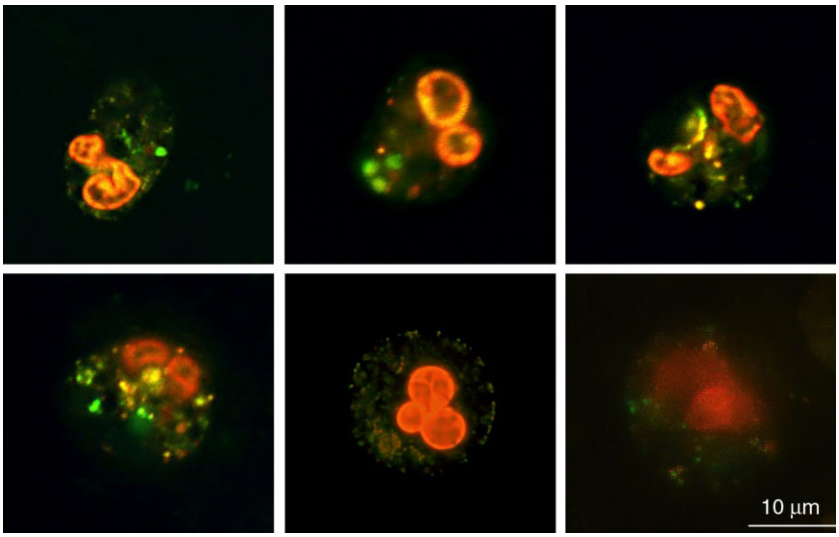

Fig. 4. Nitric oxide (NO) and superoxide (O_2^-) expression in polymorphonuclear leucocytes (PMNs) in sputum from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* lung infection. Visualization of NO and O_2^- production in PMNs in sputum by fluorescence microscopy stained with 20 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) (green colour) and 5 μM hydroethidine (red colour).

that PMNs predominate the population of NO-positive cells found in our study. Other leucocytes or other cells may have substantial effects on the NOS activity, but only PMNs are known to have the ability to employ anaerobic glycolysis to sustain production of energy [36,37] in order to provide NADPH [38], which is needed to fuel NOS activity [12]. Thus, the minor fraction of O_2 consumed in CF sputum by aerobic respiration and the resistance to treatment with KCN [5] of the luminol-enhanced chemiluminescence, which is dependent upon NOS activity [39], suggests that the influence on the NOS activity in CF sputum by cells dependent upon aerobic respiration is limited.

It is unknown how iNOS is activated in PMNs in CF sputum, although the exposure of PMNs to bacterial lipopolysaccharide (LPS), interferon (IFN)-γ and tumour necrosis factor (TNF)-α has been suggested as triggers for iNOS induction [40]. In fact, LPS, IFN-γ and TNF-α are present in CF sputum [41–43], where they have the potential to activate PMNs for both the respiratory burst and NO production.

We have previously demonstrated ongoing rapid O_2 uptake during the respiratory burst of PMNs in sputum from CF patients chronically infected with *P. aeruginosa* [5]. Both the inhibition of O_2 consumption and NO generation with L-NMMA and DAF-FM demonstrated in the present study are indications of an ongoing NO generation by the PMNs in sputum from chronically infected CF patients. The process of equilibrating the sputum samples to ambient air by dilution in Krebs–Ringer buffer is unlikely to have activated iNOS in the sputum cells, as it has been shown previously that human PMNs are not activated by resuspension in Krebs–Ringer buffer [5]. We thus argue that iNOS was already active in the endobronchial mucus prior to equilibration of the sputum samples. Our demonstration of ongoing iNOS activity in PMNs from CF sputum thus suggests strongly that iNOS in PMNs in the endobronchial mucus of CF patients converts O_2 and L-arginine to NO and L-citrulline, which contributes to the O_2 depletion in sputum. It may be argued that our results are influenced by endothelial and neuronal NOS activity, as L-NMMA inhibits the NOS subtypes with low selectivity [10], but NOS expression in neutrophils is predominated by iNOS [14,30–32]. Because NO is highly reactive and diffusible, it is recommended to apply more than one method for measuring and visualizing the production of NO [44]. In the present study, we selected methods allowing us to demonstrate that inhibition of NOS results in both reduced consumption of the substrate O_2 and reduced fluorescence of the NO indicator, DAF-FM. We found that ~28% of the O_2 consumption in infected sputum samples were used by NOS activity, which is in agreement with the O_2 consumption by NOS activity in stimulated human peripheral PMNs [9]. However, in addition to production of NO, active iNOS may also mediate the formation of minor amounts of O_2^- [12]. We have shown previously that the majority of the O_2 consumption in infected sputum is caused by the respiratory burst in PMNs, accounting for approximately 60% of the O_2 consumption in sputum [5]. The results from the present study thus corroborate that activated PMNs are the major consumers of O_2 in infected endobronchial secretions in CF.

We still need to demonstrate *in-vivo* NO production by PMNs directly in the lungs of the CF patients, and our findings do not necessarily reflect the situation of all PMNs in the endobronchial mucus of chronically infected CF patients, as the O_2 supply can vary [4]. Nevertheless, NO can persist at micromolar concentrations in solutions for several minutes before it reacts with other molecules [26], and a few preliminary measurements of NO microprofiles with NO microelectrodes in fresh sputum indicated localized concentrations of up to 4 μM NO, which was rapidly degraded. Furthermore, the large percentage of cells positive for NO generation in sputum samples strongly suggests that iNOS in PMNs was active in the lungs even before

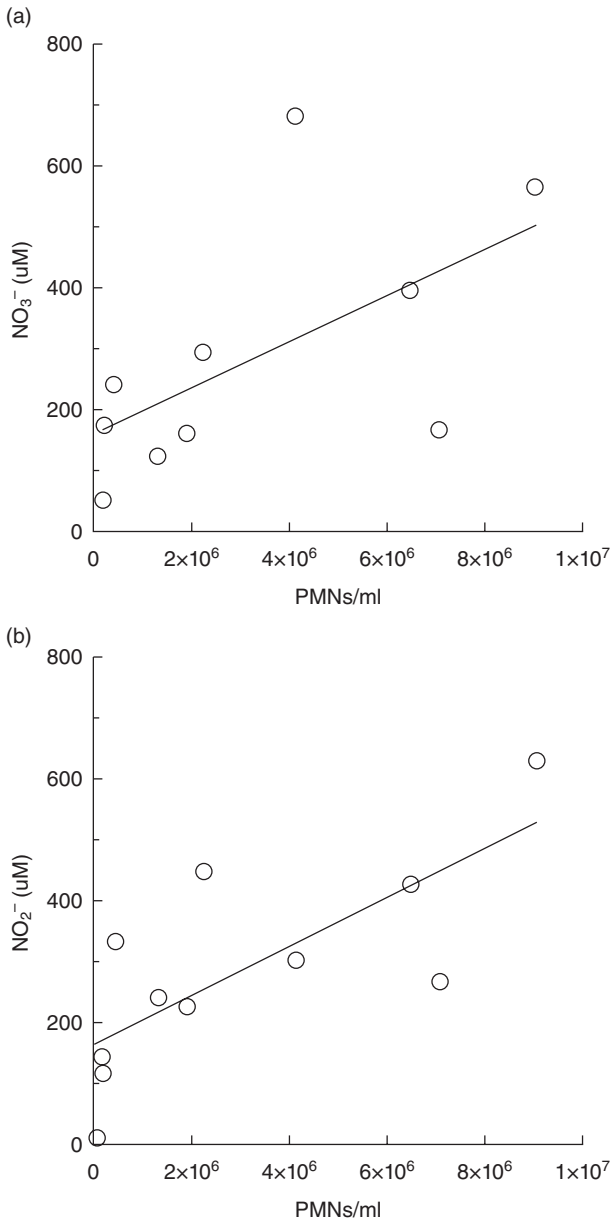

Fig. 5. Correlations between the concentration of nitrate (NO_3^-)and nitrite (NO_2^-) to the concentration of polymorphonuclear leucocytes (PMNs) in sputum from cystic fibrosis (CF) patients with chronic *Pseudomonas aeruginosa* lung infection. The concentrations of NO_3^- and NO_2^- were determined by the Griess reagent system and the concentration of PMNs was estimated by flow cytometry. (a) NO_3^- concentration *versus* the concentration of PMNs ($P < 0{\cdot}04$, $n = 10$). (b) NO_2^- concentration *versus* the concentration of PMNs ($P < 0{\cdot}006$, $n = 11$). Statistical analysis was performed by Spearman's rank correlation test.

expectoration. This implies that the iNOS in PMNs enabled the production of NO from O_2, as demonstrated experimentally [9].

To determine if sputum PMNs consume O_2 for simultaneous NO and O_2^- production, the co-expression of these reactive species in the PMNs from the sputum samples further indicates ongoing activity of both iNOS and NOX-2. The respiratory burst produces O_2^- that combines rapidly with NO to generate $ONOO^-$ [18,45]. In fact, O_2^- released by activated PMNs can thus decrease NO [46], and we suggest that NO consumption by the reaction with O_2^- contributes to the decreased levels of exhaled NO seen in CF patients [47]. In addition, nitrosative lesions in the lung tissue of CF patients can occur as a consequence of $ONOO^-$ reacting with tyrosine residues, leading to the increased content of 3-nitrotyrosine in CF lungs [48]. The combination of NO with O_2^- to generate $ONOO^-$ dramatically enhances the bactericidal effect of NO [49], and *P. aeruginosa* relies on the nitric oxide reductase (NOR) to obtain tolerance against phagocytic NO [50]. Interestingly, NOR is up-regulated in mucoid clinical isolates, which is the most virulent phenotype [7], and during hypoxic growth utilizing physiological levels of NO_3^- *P. aeruginosa* may transiently release NO in the micromolar range [51], which may contribute to the DAF-FM fluorescence that remained during inhibition with L-NMMA.

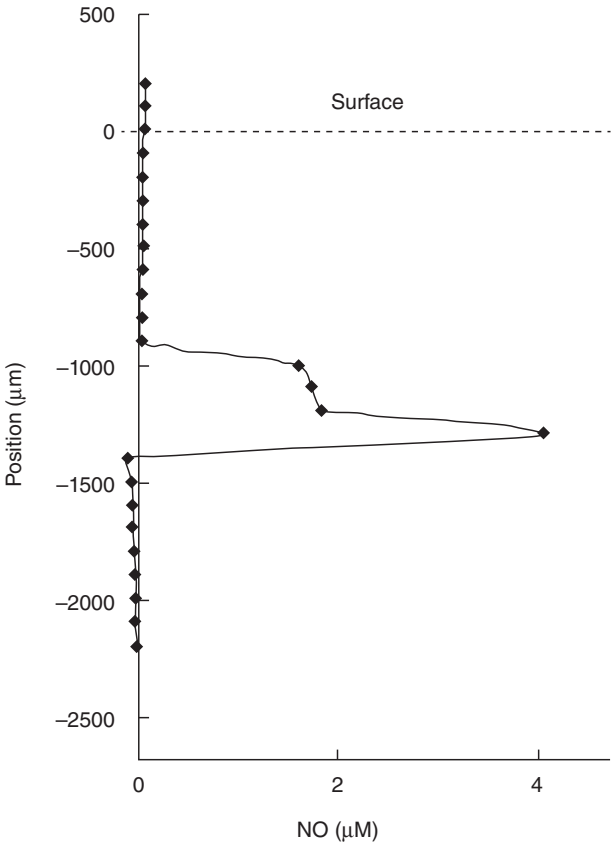

Fig. 6. Nitric oxide (NO) profile in sputum from a cystic fibrosis (CF) patient with chronic *Pseudomonas aeruginosa* lung infection. Representative microprofile of NO detected with a microsensor in CF sputum showing the local distribution of the concentration of NO.

PMNs can reduce $ONOO^-$, resulting from the reaction of NO with O_2^-, to the stable end-products NO_3^- and NO_2^- as the protonated form of ONOOH is decomposed [52]. Accordingly, our demonstration of a correlation between the density of PMNs with the concentration of NO_3^- and NO_2^- in the sputum is in agreement with the spontaneous release of NO_3^- and NO_2^- by PMNs isolated from CF sputum [53]. We therefore suggest that activated PMNs are involved in the accumulation of the NO_3^- and NO_2^- in CF sputum [47,54,55] due to the combined action of iNOS and NOX-2. Furthermore, the finding of elevated NO_3^- and NO_2^- in CF sputum samples caused by inhalation of NO [56] suggests that O_2^- from activated PMNs [5] is able to react with the inhaled NO.

Both NO_3^- and NO_2^- can function as terminal electron acceptors in bacteria either through the assimilatory pathway, where NO_3^- is reduced to ammonia, or through the dissimilatory pathway, where NO_3^- is reduced to nitrogen gas (N_2) during the denitrification process [57]. Interestingly, denitrification in the anoxic endobronchial secretions of CF patients with chronic *P. aeruginosa* lung infection is indicated by the production of nitrous oxide [6], the presence of OprF porin [58] and isolates with up-regulated genes involved in denitrification [7,59,60]. Therefore, we speculate that the activated iNOS and NOX-2 of the PMNs shapes the microenvironment in the infected CF mucus by accelerating O_2 depletion and secretion of NO_3^- and NO_2^- to a level which favours pathogens with anaerobic survival strategies such as denitrification. *P. aeruginosa*, which tolerates the assaults from activated PMNs by forming biofilm aggregates [2], is a facultative anaerobe capable of denitrification [57]. Therefore, *P. aeruginosa* is well adaptable to these changes in the lung microenvironment enforced by active PMNs. Furthermore, the microenvironment may have a profound effect on the action of antibiotics, and improvement of treatment is likely to rely on knowledge of the infectious microenvironment [61].

In conclusion, we have demonstrated significant NO production in PMNs in sputum from CF patients with chronic *P. aeruginosa* lung infection that can further deplete O_2 and facilitate growth and persistence driven by denitrification in biofilms aggregates of pathogenic bacteria, and can cause nitrosative lesions in the nearby lung tissue of the patients. Further research on nitrogen metabolism and its regulation, both in PMNs and *P. aeruginosa*, may thus gain important new insight and yield new targets for anti-microbial treatment of chronic lung infections.

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Disclosures

The authors declare no conflicts of interest.

References

1 Koch C, Høiby N. Pathogenesis of cystic fibrosis. Lancet 1993; **341**:1065–9.
2 Jensen PØ, Givskov M, Bjarnsholt T, Moser C. The immune system vs. *Pseudomonas aeruginosa* biofilms. FEMS Immunol Med Microbiol 2010; **59**:292–305.
3 Downey DG, Bell SC, Elborn JS. Neutrophils in cystic fibrosis. Thorax 2009; **64**:81–8.
4 Worlitzsch D, Tarran R, Ulrich M *et al*. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest 2002; **109**:317–25.
5 Kolpen M, Hansen CR, Bjarnsholt T *et al*. Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. Thorax 2010; **65**:57–62.
6 Kolpen M, Kühl M, Bjarnsholt T *et al*. Nitrous oxide production in sputum from cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. PLOS ONE 2014; **9**:e84353. doi:10.1371/journal.pone.0084353.
7 Lee B, Schjerling CK, Kirkby N *et al*. Mucoid *Pseudomonas aeruginosa* isolates maintain the biofilm formation capacity and the gene expression profiles during the chronic lung infection of CF patients. APMIS 2011; **119**:263–74.
8 Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 1973; **52**:741–4.
9 Carreras MC, Pargament GA, Catz SD, Poderoso JJ, Boveris A. Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. FEBS Lett 1994; **341**:65–8.
10 Wheeler MA, Smith SD, Garcia-Cardena G, Nathan CF, Weiss RM, Sessa WC. Bacterial infection induces nitric oxide synthase in human neutrophils. J Clin Invest 1997; **99**:110–6.
11 Evans TJ, Buttery LD, Carpenter A, Springall DR, Polak JM, Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. Proc Natl Acad Sci USA 1996; **93**:9553–8.
12 Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001; **357**:593–615.
13 Stuehr DJ. Enzymes of the L-arginine to nitric oxide pathway. J Nutr 2004; **134**:2748S–51S.
14 Cedergren J, Follin P, Forslund T, Lindmark M, Sundqvist T, Skogh T. Inducible nitric oxide synthase (NOS II) is constitutive in human neutrophils. APMIS 2003; **111**:963–8.
15 Kobzik L. Translating NO biology into clinical advances: still searching for the right dictionary? Am J Respir Cell Mol Biol 2009; **41**:9–13.
16 Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; **43**:109–42.
17 Arai H. Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. Front Microbiol 2011; **2**:103. doi:10.3389/fmicb.2011.00103.
18 Hassett DJ, Cuppoletti J, Trapnell B *et al*. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in

chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv Drug Deliv Rev 2002; **54**:1425–43.

19 Høiby N. Microbiology of cystic fibrosis. In: Hodson ME, Geddes DM, eds. Cystic fibrosis. London, UK: Arnold, 2000:83–107.

20 Koch C, Høiby N. Diagnosis and treatment of cystic fibrosis. Respiration 2000; **67**:239–47.

21 Pedersen SS, Jensen T, Hoiby N, Koch C, Flensborg EW. Management of *Pseudomonas aeruginosa* lung infection in Danish cystic fibrosis patients. Acta Paediatr Scand 1987; **76**: 955–61.

22 Revsbech NP. An oxygen microsensor with a guard cathode. Limnol Oceanogr 1989; **34**:474–8.

23 Kojima H, Nakatsubo N, Kikuchi K *et al.* Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 1998; **70**:2446–53.

24 Kelley TJ, Drumm ML. Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. J Clin Invest 1998; **102**:1200–7.

25 Zhao H, Kalivendi S, Zhang H *et al.* Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic Biol Med 2003; **34**:1359–68.

26 Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996; **271**:C1424–37.

27 Grasemann H, Tullis E, Ratjen F. A randomized controlled trial of inhaled l-arginine in patients with cystic fibrosis. J Cyst Fibros 2013; **12**:468–74.

28 Meng QH, Polak JM, Edgar AJ *et al.* Neutrophils enhance expression of inducible nitric oxide synthase in human normal but not cystic fibrosis bronchial epithelial cells. J Pathol 2000; **190**:126–32.

29 Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry 1988; **27**:8706–11.

30 McCall TB, Feelisch M, Palmer RM, Moncada S. Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br J Pharmacol 1991; **102**:234–8.

31 Yui Y, Hattori R, Kosuga K *et al.* Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. J Biol Chem 1991; **266**:3369–71.

32 Warner RL, Paine R, III, Christensen PJ *et al.* Lung sources and cytokine requirements for *in vivo* expression of inducible nitric oxide synthase. Am J Respir Cell Mol Biol 1995; **12**:649–61.

33 Sadeghi E, Matlow A, MacLusky I, Karmali MA. Utility of Gram stain in evaluation of sputa from patients with cystic fibrosis. J Clin Microbiol 1994; **32**:54–8.

34 Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. J Allergy Clin Immunol 1995; **95**:843–52.

35 Hector A, Jonas F, Kappler M, Feilcke M, Hartl D, Griese M. Novel method to process cystic fibrosis sputum for determination of oxidative state. Respiration 2010; **80**:393–400.

36 Sbarra AJ, Karnovsky ML. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J Biol Chem 1959; **234**:1355–62.

37 Borregaard N, Herlin T. Energy metabolism of human neutrophils during phagocytosis. J Clin Invest 1982; **70**:550–7.

38 Borregaard N, Schwartz JH, Tauber AI. Proton secretion by stimulated neutrophils. Significance of hexose monophosphate shunt activity as source of electrons and protons for the respiratory burst. J Clin Invest 1984; **74**:455–9.

39 Wang PH, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart – evidence for peroxynitrite-mediated reperfusion injury. J Biol Chem 1996; **271**:29223–30.

40 Geller DA, Billiar TR. Molecular biology of nitric oxide synthases. Cancer Metastasis Rev 1998; **17**:7–23.

41 Jensen PØ, Moser C, Kharazmi A, Pressler T, Koch C, Høiby N. Increased serum concentration of G-CSF in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* pneumonia. J Cyst Fibros 2006; **5**:145–51.

42 Kronborg G, Shand GH, Fomsgaard A, Høiby N. Lipopolysaccharide is present in immune complexes isolated from sputum in patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* lung infection. APMIS 1992; **100**:175–80.

43 Paats MS, Bergen IM, Bakker M *et al.* Cytokines in nasal lavages and plasma and their correlation with clinical parameters in cystic fibrosis. J Cyst Fibros 2013; **12**:623–9.

44 Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med 2007; **43**:645–57.

45 Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun 1993; **18**:195–9.

46 Jones KL, Bryan TW, Jinkins PA *et al.* Superoxide released from neutrophils causes a reduction in nitric oxide gas. Am J Physiol 1998; **275**:L1120–6.

47 Grasemann H, Michler E, Wallot M, Ratjen F. Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. Pediatr Pulmonol 1997; **24**:173–7.

48 Jones KL, Hegab AH, Hillman BC *et al.* Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. Pediatr Pulmonol 2000; **30**:79–85.

49 Brunelli L, Crow JP, Beckman JS. The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. Arch Biochem Biophys 1995; **316**:327–34.

50 Hoffman LR, Richardson AR, Houston LS *et al.* Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway. PLOS Pathog 2010; **6**:e1000712. doi:10.1371/journal.ppat.1000712.

51 Kakishima K, Shiratsuchi A, Taoka A, Nakanishi Y, Fukumori Y. Participation of nitric oxide reductase in survival of *Pseudomonas aeruginosa* in LPS-activated macrophages. Biochem Biophys Res Commun 2007; **355**:587–91.

52 Kissner R, Koppenol WH. Product distribution of peroxynitrite decay as a function of pH, temperature, and concentration. J Am Chem Soc 2002; **124**:234–9.

53 Francoeur C, Denis M. Nitric oxide and interleukin-8 as inflammatory components of cystic fibrosis. Inflammation 1995; **19**:587–98.

54 Hassett DJ. Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. J Bacteriol 1996; **178**:7322–5.

55 Palmer KL, Brown SA, Whiteley M. Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. J Bacteriol 2007; **189**:4449–55.

56 Ratjen F, Gartig S, Wiesemann HG, Grasemann H. Effect of inhaled nitric oxide on pulmonary function in cystic fibrosis. Respir Med 1999; **93**:579–83.
57 Zumft WG. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 1997; **61**:533–616.
58 Yoon SS, Hennigan RF, Hilliard GM *et al*. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 2002; **3**:593–603.
59 Son MS, Matthews WJ, Jr, Kang Y, Nguyen DT, Hoang TT. *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. Infect Immun 2007; **75**:5313–24.
60 Ciofu O, Johansen HK, Aanaes K *et al*. *P. aeruginosa* in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs. J Cyst Fibros 2013; **12**:729–36.
61 Bjarnsholt T, Alhede M, Alhede M *et al*. The *in vivo* biofilm. Trends Microbiol 2013; **21**:466–74.