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Oxidative Stress By Pyocyanin Impairs CFTR Cl^- Transport In Human Bronchial Epithelial Cells

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Abstract

Pyocyanin (N-methyl-1-hydroxyphenazine), a redox-active virulence factor produced by the human pathogen *Pseudomonas aeruginosa*, is known to compromise mucociliary clearance. Exposure of human bronchial epithelial cells to pyocyanin increased the rate of cellular release of H_2O_2 3-fold above the endogenous H_2O_2 production. Real-time measurements of the redox-potential of the cytosolic compartment using the redox sensor roGFP1 showed that pyocyanin (100 μM) oxidized the cytosol from a resting value of -318 ± 5 mV by 48.0 ± 4.6 mV within 2 hours; a comparable oxidation was induced by 100 μM H_2O_2. While resting Cl^- secretion was slightly activated by pyocyanin (to 10% of maximal currents), forskolin-stimulated Cl^- secretion was inhibited by 86%. The decline was linearly related to the cytosolic redox potential (1.8% inhibition/mV oxidation). CF bronchial epithelial cells homozygous for ΔF508 CFTR failed to secrete Cl^- in response to pyocyanin or H_2O_2 indicating that these oxidants specifically target CFTR and not other Cl^- conductances. Treatment with pyocyanin also decreased total cellular glutathione levels to 62% and cellular ATP levels to 46% after 24 hours. We conclude that pyocyanin is a key factor that redox cycles in the cytosol, generates H_2O_2, depletes glutathione and ATP, and impairs CFTR function in *Pseudomonas* infected lungs.

Keywords

Pseudomonas aeruginosa; Pyocyanin; Hydrogen peroxide; Oxidative stress; Intracellular redox potential; CFTR; Chloride ion transport; Cystic fibrosis; Glutathione

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INTRODUCTION

Pyocyanin (N-methyl-1-hydroxyphenazine) is a redox-active virulence factor secreted by *Pseudomonsas aeruginosa*, an opportunistic pathogen that causes pulmonary infections in hospitalized and immuno-compromised patients, and is a major pathogen associated with respiratory tract infections in cystic fibrosis (CF) [1]. Elevated levels of pyocyanin (as high as 130 μM) have been reported in pulmonary secretions of patients with CF and individuals with chronic bronchiectasis [2], as well as in ear secretions of patients with chronic otitis media [3]. Pyocyanin is secreted by most strains of *P. aeruginosa*, and its production is increased when the organism is at high density or in the biofilm form [4] as is the case in the lungs of CF patients [5]. Isolates of a CF epidemic strain of *P. aeruginosa* exhibited a widespread over-production of pyocyanin which was detectable during early exponential growth [3].

Pyocyanin is known to cause a variety of deleterious effects on airway physiology including the slow down of cilia beating [6] and mucociliary transport [7], disruption of epithelial integrity, modulation of immune protein expression [8], and an increase in IL-8 and ICAM-1 production [9]. Using a *Saccharomyces cervisiae* deletion library, 50 cellular targets for pyocyanin action were identified, including the V-type ATPase protein complex, as well as proteins involved in vesicle and protein transport, cell cycle, electron transport and respiration, and epidermal cell growth [10]. At neutral pH, pyocyanin exists as a zwitter ion that readily penetrates cell membranes, and its cytotoxic activity has been linked to its ability to generate reactive oxygen species (ROS) by redox cycling. The standard redox potential of pyocyanin (Eo'=-34 mV, [11]) is high enough to allow electron transfer from NAD(P)H (Eo'=-320 mV) and glutathione (Eo'=-240 mV) and reduced pyocyanin rapidly reacts with molecular oxygen to produce superoxide ($O_2^{\cdot -}$) which is the precursor for H_2O_2 and other ROS.

The cystic fibrosis transmembrane conductance regulator (CFTR) protein plays a key role in controlling the mucociliary clearance process in human airways [12,13] and is mutated in CF. CFTR functions as a cAMP-regulated chloride and HCO_3 channel [14], is permeable to other small anions [15], and provides a pathway for the exit of reduced and oxidized glutathione [16,17]. There is emerging evidence that reactive oxygen and nitrogen species target the activity of CFTR [18-21]. Patch clamp studies demonstrated that oxidizing conditions slow CFTR channel gating, presumably via cysteine residues that are present in the two nucleotide-binding domains and the regulatory domain of CFTR [19,21]. Oxidized forms of glutathione inhibited CFTR channels by a mechanism that involved the glutathionylation of a cysteine residue at position Cys-1344 of the CFTR molecule [22]. Measurements of transepithelial Cl^- secretion in Ussing chambers suggested that the CFTR-mediated Cl^- secretion is affected by redox-active reagents, though some studies show activation by H_2O_2 [23-26], hypochlorus acid [27] and isoprostanes [28], whereas others report inhibition by H_2O_2 [26,29], *tert*-butylhydroquinone [30], and cigarette smoke extract [31,32]. A recent study dissected the dual effects of H_2O_2 and reported that secretion was stimulated by both apically and basolaterally applied H_2O_2 under resting conditions, whereas basolaterally but not apically applied H_2O_2 potentiated cAMP-stimulated anion secretion [26]. Other evidence for reduced CFTR activity by ROS comes from nasal potential difference measurements in smokers that suggested an acquired CFTR deficiency in the respiratory epithelium [31].

The role of *Pseudomonas aeruginosa* infection on CFTR has recently been studied and two reports suggested an inhibiton. A cell-free filtrate of P. *aeruginosa* (PA14) reduced CFTR Cl^- transport by inhibiting the endocytic recycling of CFTR [33] and pyocyanin altered CFTR trafficking presumably through inhibition of the organelle H^+ V-type ATPase [34].

We hypothesized that pyocyanin, through its ability to redox cycle and produce H_2O_2, would affect CFTR-mediated Cl^- transport in bronchial epithelial cells. We have previously shown

that the intracellular redox-state is not affected by the expression of wildtype CFTR [35]. In this study we determined the effects of pyocyanin on both CFTR Cl^- transport and real-time oxidation of the cytosolic compartment by image-analysis of a redox-sensitive GFP (roGFP1; [35-37]). Combining these techniques yielded quantitative information about the impact of pyocyanin-induced oxidative stress on CFTR activity and intracellular redox potential. The involvement of CFTR was determined by comparing the Cl^- secretory responses to pyocyanin and H_2O_2 in CFTR-corrected *vs.* parent CFBE41o- cells homozygous for ΔF508 CFTR as a negative control. Results obtained here suggest that pyocyanin exerted a small stimulatory effect on resting CFTR activity, and a large inhibitory effect on cAMP-stimulated CFTR activity with slow kinetics through a mechanism that involved intracellular oxidation and other factors. Exposure of the apical membrane of CFTR-corrected CFBE41o-monolayers to pyocyanin decreased total cellular GSH by 38% and intracellular ATP by 54% of untreated controls within 24 hours.

MATERIALS AND METHODS

Reagents

Unless otherwise specified, reagents and chemicals were obtained from Sigma (St. Louis, MO). The adenylate cyclase activator forskolin (Calbiochem, La Jolla, CA) was prepared in DMSO as a 20 mM stock solution and used at a final concentration of 20 μM added to the basolateral side; the CFTR-blocker GlyH101 [38] was kindly provided by Drs Nitin Sonawane and Alan Verkman (Univ. of California, San Francisco) or synthesized by ourselves (ADM & MJK) and was prepared as a 20 mM stock in DMSO. Pyocyanin was synthesized as described below or was obtained from Colour Your Enzyme (Toronto, Ontario, Canada), and used from a 10 or 100 mM stock solution in deionized water. Diphenylene iodonium (DPI) was made as a 5 mM stock and used at 1 μM.

Synthesis of pyocyanin

1-Hydroxyphenazine (0.100 g, 0.510 mmol, American Tokyo Kasie, Portland, OR) was added to methyl sulfate (0.579g, 4.918 mmol) and the solution was heated to 100 °C for 10 min in a flask fitted with a calcium chloride drying tube. The solution was allowed to cool to room temperature and dry ether was added (4 ml). A dark brown solid precipitated, was filtered, and washed with 3 portions of dry ether (10 ml). The solid was dissolved in water and made alkaline with several drops of a 10% sodium hydroxide solution. Once added, the solution changed color from a dark brown to a dark blue color. The aqueous layer was extracted exhaustively with chloroform until the organic layer was free of the blue pyocyanin compound. The chloroform extracts were combined and washed with three portions of a 5% hydrochloric acid solution (10 ml). The aqueous layer from the acid washes was made alkaline with 10% sodium hydroxide and extracted with chloroform exhaustively as described previously. The organic layer was dried over sodium sulfate and concentrated. Pyocyanin was recrystallized by dissolving the blue solid in the least amount of water possible and allowing the solution to cool in an ice bath. The crystals were collected and gave pyocyanin as a bright blue solid. Melting point was determined on a Fisher-Johns apparatus and was 131°C (reported 133°C [39]). ^1H NMR and IR were recorded in CDCl3 with solvent signals used for reference at 7.26 ppm for 1H and 77.16 for 13C NMR and were consistent with published results [40]. Purity of pyocyanin was > 95% as determined by LCMS, which was accomplished using a Waters Alliance equipped with a Nova-Pak C18 column (3.9 × 150 mm).

Human bronchial epithelial cell culture

The parent CFBE41o- cell line was originally derived from a bronchial tissue isolate from a CF patient homozygous for the ΔF508 CFTR mutation and immortalized with the pSVori- plasmid that contained a replication-deficient simian virus 40 (SV40) genome. For the

generation of corresponding CFTR-complemented line, the parental CFBE41o- cell line was transfected with an Epstein-Barr virus (EBV) based episomal expression vector pCEP4 (Invitrogen) containing normal CFTR cDNA via electroporation (Nucleofector II, Amaxa Biosystems). The construct comprised the entire ~6.2 kb CFTR cDNA that contained both the open reading frame and the 5' and 3' untranslated regions. CFTR-CFBE41o- cells were cultured in Minimum Essential Medium (Invitrogen) containing 10% fetal bovine serum (HyClone), 2 mM L-glutamine (UCSF cell culture facility), 100 unit/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and were kept under selective pressure using 300 μg/ml hygromycin B (Invitrogen). The culture flasks and permeable filter inserts were coated with an extracellular matrix cocktail comprised of 0.01 mg/ml human fibronectin (BD Biosciences), 0.029 mg/ml Vitrogen (Cohesion, Inc.), and 0.1 mg/ml bovine serum albumin (Biosource/Biofluids). For transepithelial measurements, cells were seeded on Snapwell inserts (0.4-μm pore size, 1.1 cm^2; Costar, Cambridge, MA) at a density of 5×10^5 cells/filter. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Both the parental and CFTR-corrected CFBE41o- cell lines consistently maintained an epithelial phenotype and formed moderately tight epithelial monolayers, expressed Ca-activated Cl^- currents, but no amiloride-sensitive Na absorption, and the CFTR-CFBE41o- clone maintained a consistently high level of transgene expression over all observed passages [41].

Measurement of cellular H_2O_2 production

H_2O_2 production was measured using the Amplex Red assay kit (Molecular Probes, USA). CFBE41o- cells were grown to confluency in 96-well plates (0.316 cm^2 epithelial area, 100 μl fluid volume) and washed twice with glucose-supplemented PBS to remove the cell medium before the assay. Cells were exposed to increasing concentrations of H_2O_2 in glucose-supplemented PBS at a final volume of 100 μl. Amplex Red reacts in a 1:1 stoichiometry with H_2O_2 to produce resurofin, which was detected by a fluorescence plate reader (excitation, 530 ± 20 nm,; emission, 590 ± 10 nm, Wallac Victor^2, Perkin Elmer). Fluorescence was measured every 30 min and calibrated against a standard curve generated from serial dilutions of H_2O_2. Rates of epithelial H_2O_2 production (in $nmole\cdot h^{-1}\cdot cm^{-2}$) were determined from slopes of calculated H_2O_2 concentrations *vs.* time by linear regression. Experiments were performed at least in quadruplicates.

Redox potential measurements using roGFP1 and imaging microscopy

Measurements of cytosolic redox potentials were performed in CFTR-CFB41o- cells transiently expressing redox-sensitive GFP mutant exactly as described [35]. Approximately 100,000 cells were exposed to 100 μM pyocyanin or increasing concentrations of H_2O_2 in a fluid volume of 100 μl. Briefly, cells were grown on cover glasses and transiently transfected with a plasmid coding for a redox-sensitive GFP mutant (roGFP1 [36,37]) using Effectene transfection reagent (Qiagen). After 24 to 48 hours, roGFP1-expressing cells were mounted in a chamber on the stage of a Nikon Diaphot microscope with a 40x Neofluar objective (1.4 NA) and bathed in 100 μl Hepes-buffered Ringer's solution. Ratiometric imaging was performed using a CCD camera, filter wheel (Lambda-10, Sutter Instruments) and Axon Imaging Workbench 4 (Axon CNS Molecular Devices) to collect emission (>510 nm) images during alternate excitation at 385 ± 5 nm and 474 ± 5 nm. Background-subtracted roGFP1 fluorescence ratios were recorded, and at the end of each experiment, maximal oxidation by treatment with 10 mM H_2O_2 and maximal reduction by treatment with 10 mM DTT was performed to determine the total dynamic range of the dye. Fluorescence ratios were averaged and converted to redox potentials (in mV) using a published *in situ* calibration curve [35].

Transepithelial short-circuit current measurements

CFBE41o- monolayers grown on Snapwell cell culture inserts were mounted via a slider into a modified Ussing chamber (Easy Mount Chamber Systems, Physiologic Instruments, San Diego, CA). Approximately 1×10^6 cells were exposed to 100 μM pyocyanin or H_2O_2 in a fluid volume of 5 ml. Transepithelial experiments were performed as previously described [42]. A serosal-to-mucosal Cl^- gradient was used to increase the electrochemical driving force for Cl^- secretion. The basolateral Ussing chamber solution contained (in mM): 120 NaCl, 20 $NaHCO_3$, 5 $KHCO_3$, 1.2 NaH_2PO_4, 5.6 glucose, 2.5 $CaCl_2$, and 1.2 $MgCl_2$. In the mucosal solution, all Cl^- salts were exchanged for gluconate salts. Both hemi-chambers were constantly gassed with 5% CO_2 /air and maintained at 35-37°C.

LC/MS/MS detection of GSH and GSSG

CFTR-CFBE41o- monolayers were grown to confluency on cell culture inserts (Falcon, 0.4-μm pore, 1.0 cm^2; BD Labware, Franklin Lakes, NJ) and exposed to 1 ml cell culture medium containing 100 μM pyocyanin from the apical side for 24 hours. Intracellular GSH and GSSG were detected using LC/MS/MS following derivatization with isopropylchloroformate based on methods previously published by Husek et al. [43] with the following modifications. Membranes were cut from the inserts and placed into 500 μL volume of 10 % perchloric acid (PCA) containing 1 mM DTPA and frozen at −80°C. From these samples, the acid-soluble supernatants were collected following centrifugation at 14,000 rpm for 5 min. To the acidified supernatants, homoglutathione (10 μM) was added as an internal control. Subsequent to the addition of internal standards, solid phase extractions with a strong cation exchange resin (phenomenex) were performed to further enrich GSH and GSSG. GSH and GSSG were eluted by treating with 100 μl elution buffer consisting of 0.1 N NaOH, 40% N-propanol, and 10% pyridine. The eluted samples were mixed with 50 μl of derivatizing solution consisting of isooctane, chloroform, and isopropylchloroformate (75:40:10) and allowed to react for 2 min. The derivatized products were subsequently extracted with 200 μl of isooctane and dried under a constant stream of nitrogen. These samples were reconstituted with mobile phase consisting of methanol and water (80:20, v/v). Chromatographic separation of GSH, GSSG, and homoglutathione were performed on C18 reversed-phase column (25 × 2 mm, 0.3 μm) from Phenomenex (Torrance, Ca). The chromatographic system was Shimadzu LC-10AV separation module. The separation was performed under isocratic condition at a flow rate of 0.2 ml/min. Electrospray tandem mass spectrometric analysis was performed on Quattro Micro mass spectrometer from Micromass and analyzed using Masslinks software (3.1 version). Analytes were detected using the multiple reaction monitoring (MRM) scanning mode. Capillary voltage was set to 3 kV, source temperature to 150 C and nebulizer gas temperature to 400 C. The cone and evaporation gas flows were set at 200 and 800L/hr, respectively. Cone voltages for GSH, GSSG, and homoglutathione were set 35 V, 58 V, and 35 V, respectively. Collison energy for GSH, GSSG, and homoglutathione were all set at 20 e. Mass transition pairs monitored for GSH, GSSG and homoglutathione derivatives were 564.5>162.2, 953.5>836.5, and 578.5>162.2. Homoglutathione was used as internal standards for quantification of the GSH/GSSG redox couple. Absolute concentrations were calculated relative to cell number and a cell volume (5 pl per cell).

ATP measurements

Cellular ATP levels were determined using a a bioluminescent ATP assay kit according to the manufacturers instructions (EnzyLight™ ATP assay kit, BioAssay Systems, Hayward, CA). CFTR-corrected CFBE41o- cells were plated on a white, clear bottom 96-well plates (Costar 3610, Corning Incorporated, Corning, NY), grown to confluency and exposed to 100 μM pyocyanin in 100 μl cell culture medium for 24 hours. At the end of the incubation, cells were lysed with 90 μl assay buffer containing substrate (D-luciferin) and ATP enzyme (luciferase).

ATP was determined by the amount of light emitted after the reaction of D-luciferin and ATP catalyzed by luciferase. The luminescence signal was measured within 20 minutes using the ATP luminometry mode of the plate reader (Wallac Victor^2, Perkin Elmer) with an integration time of 1.0 sec and calibrated to a standard curve. ATP levels were reported in μM per well (0.316 cm^2) and average values were calculated from n = 8 wells in each group.

Statistical analysis

Data are presented as original values or as mean ± SE; n refers to the number of experiments. Linear regressions and Hill-Michaelis-Menten fits as described in [42] were done with Sigmaplot (version 10, Systat Software). Effects of treatment were tested using t tests. Comparisons of multiple groups were done by ANOVAs followed by pairwise comparisons using the Holm-Sidak method. Statistical calculations were done using SigmaStat (version 3.5, Systat Software). Resulting p values are given and $p<0.05$ was considered significant.

RESULTS

Pyocyanin enhances cellular production of H_2O_2 in bronchial epithelial cells

Pyocyanin is known to generate reactive oxygen species and H_2O_2 by its ability to redox cycle with cellular electron donors, such as intracellular NADPH and glutathione [44,45]. First we tested basal and pyocyanin-induced H_2O_2 release by human CF bronchial epithelial cells (CFBE41o-). Cells grown in 96-well plates were treated with increasing concentrations of pyocyanin and the release of H_2O_2 into the extracellular buffer was measured using the Amplex Red reagent. Fig. 1A shows the time- and concentration-dependent effects of pyocyanin on cellular H_2O_2 production. These data were used to calculate rates of H_2O_2 release for every pyocyanin concentration by linear regression. Corresponding rates of pyocyanin-induced H_2O_2 release followed saturation kinetics (Fig. 1B) with a maximal rate of 1.67 ± 0.11 nmole·h^{-1}·cm^{-2}, and a half-maximal concentration of pyocyanin of 21.1 ± 2.0 μM. Because airway epithelial cells have their own mechanism to produce H_2O_2, which is governed by dual NADPH oxidases (Duox) [46], we used the NADPH oxidase blocker diphenylene iodinium (DPI, 1 μM) to distinguish Duox-derived from pyocyanin-induced H_2O_2 production. CFBE41o- cells generated H_2O_2 at a rate of 0.45 ± 0.03 nmole·h^{-1}·cm^{-2} (Fig. 1C, grey squares), and 0.31 ± 0.03 nmole·h^{-1}·cm^{-2} (n=25) were blocked by DPI (Fig. 1C, open squares). Treatment with pyocyanin (100 μM; Fig. 1C, filled circles) increased cellular H_2O_2 production 3-fold to 1.35 ± 0.03 nmole·h^{-1}·cm^{-2}, and 0.48 ± 0.03 nmole·h^{-1}·cm^{-2} (n=3 were blocked by DPI (Fig. 1C, open circles). Effects of DPI were not different in pyocyanin-treated CFBE41o- cells.

Next we tested the ability of NADPH and GSH to redox cycle and produce H_2O_2 in presence of pyocyanin in cell free experiments. Both NADPH and GSH have previously been shown to serve as electron donors for pyocyanin [45] and this set of experiments was to quantify their relative contributions and verify their effects in our experimental system. Pyocyanin alone had no measurable effect on the Amplex Red signal in cell free experiments and required the presence of an extracellular reductant. Pyocyanin (100 μM) in presence of NADPH (150 μM) generated H_2O_2 at a rate of 3.49±0.37 nmole/h (n=4). For comparison, GSH (150 μM) generated H_2O_2 under the same conditions at a significantly lower rate of 0.115±0.064 nmole/h (n=4). The time-dependent and dose-dependent H_2O_2 production of NADPH and GSH with 100 μM pyocyanin in cell free experiments is shown in Figs. 1D&E. The H_2O_2 production in presence of NADPH increased with dose and time and saturated the detection within minutes at concentrations larger than 1 mM (Fig. 1D). GSH showed increased H_2O_2 production over time but the concentration dependence showed a peak at 150 μM. At higher concentrations the detected H_2O_2 concentrations decreased greatly (Fig. 1E). A possible explanation is that high millimolar concentrations of GSH reduce the concentration of H_2O_2 available for detection by Amplex Red (as similarly found for anthracyclines [47]). Despite this possible limitation of

this assay, it is apparent that both NADPH and GSH redox cycle with pyocyanin at different rates.

These measurements indicated that i) exposure of CFBE41o- cells to pyocyanin enhanced the generation of H_2O_2 independent of and additive to the basal H_2O_2 production, and ii) the intracellular electron donors NADPH and GSH redox cycle with pyocyanin suggesting ready entry of pyocyanin into cells.

Real-time measurements of cytosolic oxidation by pyocyanin and H_2O_2

The acute effect of pyocyanin on intracellular oxidation was measured using a cytosolic redox-sensitive GFP mutant (roGFP1) that was recombinantly expressed in CFTR-corrected CFBE41o- cells (CFTR-CFBE41o-) and compared to effects of treatment with H_2O_2. Typical pseudocolor fluorescence ratio images of CFTR-CFB41o- cells expressing roGFP1 are shown in Figs. 2A-D. The resting redox potential of the cytosol was uniformly low and reduced (Fig. 2A,C) averaging -318 ± 5 mV (n = 6), similar to previous results obtained in the CF nasal epithelial cell line CF15 [35]. Exposure to pyocyanin (100 μM) oxidized the cytosol considerably after 2 hours (Fig. 2B), and H_2O_2 (100 μM) induced a similar oxidation already after 20 min (Fig. 2D). Fig. 2E shows the time-dependent oxidation of the cytosolic redox potential (E_{Redox}) induced by 100 μM pyocyanin and by increasing concentrations of H_2O_2 from 10 μM to 500 μM. Notably, addition of defined concentrations of H_2O_2 resulted in rapid cytosolic oxidations that reached a relatively stable plateau within minutes, and step-wise increases of [H_2O_2] resulted in step-wise oxidations of redox potentials, consistent with the notion that H_2O_2 entered the cell and oxidized its targets. The stable E_{Redox} attained after addition of H_2O_2 suggested that cellular reducing mechanisms were overwhelmed by the relatively large amounts of H_2O_2 added since we previously found in comparable measurements that cells readily reduced E_{Redox} when H_2O_2 was removed [48, 49]. E_{Redox} was maximally oxidized to about -255 mV at 500 μM H_2O_2, at which point roGFP1 saturates and is completely oxidized [35]. In contrast, addition of pyocyanin caused a slow and steady oxidation of the cytosol suggesting a constant redox cycling reaction and oxidation analogous to the continuous H_2O_2 release into the extracellular medium (Fig. 1A). These measurements showed that exposure to 100 μM pyocyanin for a period of 2 hours caused an oxidation of the cytosolic compartment by 48.0 ± 4.6 mV (n=3), a value that was similar to the effects of 100 μM H_2O_2 (48.1 ± 4.5 mM, n=3; Fig. 2F). This supports the notion that pyocyanin redox cycles with an intracellular electron donor resulting in intracellular oxidation and release of H_2O_2. These data also suggest that pyocyanin enters the cell quickly since both intracellular oxidation and extracellular H_2O_2 release is detected within minutes (compare Figs. 1C and 2E).

Activation of CFTR Cl^- currents by pyocyanin in resting cells

CFTR is activated in cells by hormone- and neurotransmitter-induced cAMP signaling, and the protein kinase A-associated phosphorylation/dephosphorylation is the most thoroughly understood process that regulates CFTR activity. However, there is increasing evidence that other mechanisms modulate the activity of CFTR under certain cellular conditions. To examine the roles of pyocyanin and H_2O_2 on CFTR function we used the Ussing chamber assay and measured Cl^- secretory responses in CF bronchial epithelial cells homozygous for ΔF508 CFTR (CFBE41o-) and CFTR-corrected CF bronchial epithelial cells (CFTR-CFBE41o-). Acute exposure of the apical membrane to pyocyanin (100 μM) activated resting Cl^- currents in CFTR-corrected (Fig. 3A) but not in CF bronchial epithelial cells (Fig. 3B) suggesting that pyocyanin stimulated CFTR-mediated Cl^- secretion. The pyocyanin-stimulated Cl^- current (I_{Cl}) was completely blocked by the CFTR inhibitor GlyH101 in CFTR-corrected cells (Fig. 3A) but had no effect in CF cells (Fig. 3B). As a positive control, subsequent addition of ATP (500 μM) to the apical Ussing reservoir elicited a Cl^- secretory response in both CFTR-corrected and CF cells, as well as in the presence of GlyH101 (Figs. 3A,B) indicating similar

responsiveness of these cell lines to an unrelated agonist that activates the calcium-activated chloride conductance. Acute exposure of the apical membrane to H_2O_2 (100 μM) induced Cl^- secretion in CFTR-corrected (Fig. 3C) but not in CF cells (Fig. 3D), and the H_2O_2-stimulated I_{Cl} was completely blocked by GlyH101. The pyocyanin-stimulated CFTR Cl^- current averaged 7.3 ± 0.9 μA/cm^2 (n=11) and was small compared to stimulation by 100 μM H_2O_2 (47.4 ± 5.8 μA/cm^2, n=9) or the cAMP-elevating agonist forskolin (67.4 ± 5.3 μA/cm^2, n=34) (Fig. 3E). For comparison, ATP-stimulated Cl^- currents peaked at similar values for both CFTR-corrected and CF bronchial epithelial cells (on average, 26.5 ± 4.6 μA/cm^2, n=23; Fig. 3E).

If the effects of pyocyanin on CFTR-dependent I_{Cl} were mediated by H_2O_2 production, it was expected that H_2O_2 would have similar stimulatory effects on I_{Cl} and E_{Redox}. Fig. 4A&B show the acute (first four mins) effects of treatment of CFTR-corrected CF bronchial epithelial cells with 10 μM H_2O_2 or 100 μM pyocyanin on I_{Cl} measured in Ussing chambers (Fig. 4A) and on the cytosolic E_{Redox} measured by fluorescence microscopy (Fig. 4B) using identical protocols for either experimental approach. Treatment of cells with 100 μM pyocyanin resulted in effects similar to those elicited by 10 μM H_2O_2 (Figs. 4A&B). Changes in cytosolic E_{Redox} and I_{Cl} correlated tightly (Fig. 4C), resulting in a relation of 1.41 ± 0.05 μA/cm^2 current stimulation per mV of cytosolic oxidation with no apparent difference between the regressions for the effects of H_2O_2 and pyocyanin (t test, p=0.21).

Inhibition of cAMP-stimulated CFTR Cl^- currents by pyocyanin

Next we determined the effects of pyocyanin on CFTR Cl^- currents under cAMP-stimulated conditions. CFTR Cl^- currents were stimulated by the cAMP elevating agonist forskolin (20 μM), and subsequent addition of pyocyanin caused a gradual decline of the forskolin-stimulated I_{Cl} (cAMP-I_{Cl}) over the course of 2-3 hours (Fig. 5A). A small fraction of the remaining I_{Cl} was inhibited by GlyH101. Wash-out of pyocyanin after a 2-hr incubation partially restored CFTR Cl^- currents to ~40% (not shown) suggesting that CFTR transport is not irretrievably damaged by pyocyanin. A similar inhibitory effect on cAMP-I_{Cl} was observed when 100 μM H_2O_2 was added to the apical reservoir (Fig. 5B). In a corresponding time control experiment the spontaneous decline of the cAMP-I_{Cl} was measured (Fig. 5C). In untreated monolayers cAMP-I_{Cl} remained stimulated during a 2-3 hours period, whereas exposure to either pyocyanin or H_2O_2 significantly inhibited cAMP-I_{Cl} by 86.0 ± 5.8% (n=8) and 40.0 ± 2.7% (n=4) respectively (Fig. 5D). A similar inhibitory effect of pyocyanin was observed on CFTR Cl^- currents that were stimulated by the cAMP analogue 8-(4-chlorophenylthio)-adenosine (100 μM; 78±5% inhibition, n=2) or the CFTR activator genistein (20 μM; 74±4% inhibition, n=2) suggesting that the mode of inhibition dose not involve effects of proximal CFTR regulators. The observed pyocyanin-induced inhibition of CFTR has important implication for the restoration of CFTR function in infected airways of CF patients.

Time course comparisons of the inhibitory effects of pyocyanin and H_2O_2 (both 100 μM) on cytosolic oxidation and forskolin-stimulated I_{Cl} are shown in Figures 5E-G. The pyocyanin-induced inhibition of cAMP-I_{Cl} occurred with a slow time course similar to the oxidation of the cytosol, and the initial rate of oxidation averaged 0.57 mV/min (Fig. 5E). In contrast, H_2O_2 caused a ten times faster oxidation with an initial rate of 5.9 mV/min. E_{Redox} stabilized within 20 minutes and the decline of cAMP-I_{Cl} by H_2O_2 reached stable values after 60 min (Fig. 5F). Both pyocyanin and H_2O_2 (at 100 μM each) elicited similar oxidations of the cytosolic redox potentials after two hours of treatment. However, the effect of pyoycanin on cAMP-I_{Cl} was significantly larger than that of H_2O_2 (ANOVA, p<0.001, Fig. 5D) suggesting that pyocyanin generated H_2O_2 and had additional effects that inactivated CFTR. This is further supported by the data shown in Fig. 5H, where the remaining portion of the cAMP-I_{Cl} is plotted *vs*. the corresponding E_{Redox}, i.e., the time-independent relation between decline in forskolin-

stimulated Cl^- current and degree of cytosolic oxidation. The inhibition of cAMP-I_{Cl} shows significantly different dependencies on E_{Redox} when treated with either pyocyanin or H_2O_2 (100 μM each). Treatment with pyocyanin blocked 1.81 ± 0.1% of I_{Cl} per mV change of E_{Redox}, while treatment with H_2O_2 blocked 0.75 ± 0.07% of I_{Cl} per mV change of E_{Redox} ($p<0.001$). This indicated a significantly stronger inhibition of cAMP-stimulated CFTR activity by pyocyanin than by H_2O_2 at comparably oxidized redox potentials.

Chronic exposure to pyocyanin results in loss of total cellular GSH

Measurements of the cytosolic redox potential using RoGFP1 showed that chronic exposure to pyocyanin shifted E_{Redox} towards oxidation by 48.0 ± 4.6 mV, and we further determined whether this change in E_{Redox} caused alterations in the intracellular levels of glutathione (GSH) and glutathione disulfide (GSSG). Incubation of the apical cell membrane of CFTR-corrected CFBE41o- cells (grown on 1 cm^2 filter inserts) with 1 ml cell culture medium containing 100 μM pyocyanin over a period of 24 hours resulted in a significant decrease of total cellular GSH levels to 62% of untreated control cells. Assuming a cell volume of 5 pl and unchanged volumes during pyocyanin exposure, we estimate that the total cellular GSH concentration dropped from 2.7 mM to 1.7 mM. (Fig. 6A). Determination of intracellular levels of GSSG showed that pyocyanin did not produce a significant increase in GSSG levels in CFTR-CFBE41o- cells (Fig. 6B) and the GSH/GSSG redox couple was not significantly changed (Fig. 6C) suggesting that pyoycanin depleted the intracellular antioxidant defense.

Because CFTR-mediated Cl secretion is dependent on intracellular ATP levels and pyocyanin is known to deplete cells of ATP [50,51] we further investigated the effects of treatment of CFBE41o- cells with 100 μM pyocyanin on intracellular ATP levels. Fig. 6D illustrates that pyocyanin decreased intracellular ATP levels to 46% within 24 hours. A reduction in intracellualr ATP levels is expected to significantly impair CFTR function [52,53]. Thus, treatment of cells with pyocyanin resulted in significant reduction in intracellular levels of both GSH and ATP.

DISCUSSION

The present study showed that physiologically relevant concentrations of pyocyanin resulted in 1) increased H_2O_2 production by bronchial epithelial cells to 3-fold above endogenous rates, 2) oxidation of the cytosol by −48 mV, 3) rapid stimulation of resting CFTR Cl^- currents, 4) long-term inhibition of cyclic AMP-activated CFTR Cl^- currents, 5) loss of ~1/3 of total cellular GSH, and 6) loss of ~1/2 of cellular ATP. The measured Cl^- currents were identified as CFTR-mediated by comparing Cl^- secretion in CF *vs*. CFTR-corrected bronchial epithelial cells and by inhibition with the CFTR blocker GlyH101. The observed stimulation and inhibition of CFTR by pyocyanin were kinetically distinct, of different magnitude, and showed differential dependence on the intracellular redox potential indicating that distinct cellular mechanisms were affected by pyocyanin.

Activation of CFTR by pyocyanin

Addition of pyocyanin to resting cells rapidly (maximal stimulation within 3-5 min) activated CFTR-mediated Cl^- currents (7.3 ± 0.9 μA/cm^2) to 9.3% of forskolin-stimulated currents (78 ± 2.5 μA/cm^2). The initial effect of pyocyanin on the stimulation of CFTR and E_{Redox} was mimicked by addition of 10 μM H_2O_2 suggesting that pyocyanin elicited its effects through cellular production of H_2O_2. The pyocyanin and H_2O_2-induced stimulation of CFTR-mediated Cl^- currents in CFTR-corrected CF cells is consistent with a previous study in Calu-3 cells [24,26] that reported a rapid DPC-blockable increase in transepithelial Cl^- secretion by H_2O_2. Notably, the H_2O_2 concentrations previously used to stimulate CFTR Cl^- currents in Calu-3 cells were 10 to 50-times higher when compared to CFTR-corrected CFBE41o- cells

under similar experimental conditions (1-5 mM, [24,26]). Since the recording conditions between these studies were similar, this suggests a cell type-dependent sensitivity to H_2O_2 treatment.

Oxidation of the cytosolic redox potential correlated tightly with activation of CFTR Cl^- currents during the first few minutes of treatment with either pyocyanin or H_2O_2 (Fig. 4C). Taken together with data showing pyocyanin stimulated H_2O_2 production by cells, these data indicated that the effects of pyocyanin on CFTR resulted from pyocyanin-induced production of cellular H_2O_2 by redox cycling, which oxidized the cytosol and activated CFTR. Whether this is a direct or indirect effect on CFTR is currently unclear because both CFTR itself [20, 21] and its major regulators (PKA, phosphatases [54]) have been shown to be redox sensitive.

Inhibition of CFTR by pyocyanin

Unlike the rapid onset for the stimulation of CFTR activity by pyocyanin, its inhibition of forskolin-stimulated CFTR was characterized by its markedly slower kinetics, where maximal inhibitory effects were reached only after 2-3 hours. Some of the CFTR inhibition likely resulted from pyocyanin-induced oxidation, as shown by the inhibitory effects of H_2O_2-induced oxidation. Previous studies showed that, oxidative stress (by treatment with *tert*-butylhydroquinone) reduced CFTR expression by increasing the rate of mRNA degradation in T84 cells [30]. In addition, the V-type H^+ ATPase in airway cells was inhibited by pyocyanin, resulting in reduced trafficking of CFTR to the plasma membrane [34]. The slow kinetics of CFTR inactivation that we have observed after pyocyanin treatment appears consistent with these observations.

Our data also suggest that the inhibitory effect of pyocyanin on CFTR Cl^- currents includes an additional non-redox-mediated component: although both pyocyanin and H_2O_2 inhibited forskolin-stimulated CFTR, the effect of pyocyanin was larger (pyocyanin blocked 86% of CFTR current compared to 40% by H_2O_2, Fig. 5D), and its relation to the cytosolic redox potential was steeper (1.8% change of current per mV) compared to H_2O_2 (0.75% change of current per mV; Fig. 5H). Thus, the block of CFTR by pyocyanin was only partially mimicked by the addition of H_2O_2 despite similar effects on the cytosolic redox potential. This suggests that pyocyanin exerted inhibitory effects on CFTR Cl^- currents that involved additional cellular targets, as proposed previously for the inhibition of catalase [55], ciliary beating [6] and cytoplasmic ATP concentrations [50]), although some of these observations may have resulted through indirect redox effects of pyocyanin. Previous determinations of whether an observed effect was redox-dependent or redox-independent have relied largely on experimental maneuvers that mimic oxidation (e.g., by addition of H_2O_2) or that inhibits oxidation (e.g., by addition of catalase). In our study we continuously measured cytosolic redox potentials and Cl^- currents during the experimental manipulations, which allowed us to quantitatively relate the redox potentials to CFTR Cl^- currents. Based on this approach (Fig. 5H) we conclude that <50% of pyocyanin-inhibition of CFTR occurred through an oxidation-mediated effect that was mimicked by H_2O_2-induced oxidation, while a large fraction (>50%) of pyocyanin's inhibitory effect may be governed by other effects.

We found that treatment of CFBE41o- cells with pyocyanin results in a significant decrease of ATP concentrations, which was also previously noted [50]. In this regard, it is important to consider the involvement of the AMP-activated protein kinase (AMPK) which is a ubiquitous metabolic sensor that responds to small changes in intracellular ATP levels. Also, CFTR has the unique property that it requires ATP for its gating suggesting that its activity is coupled to the cellular metabolic state. AMPK was discovered to interact with CFTR and inhibited its activity in transepithelial experiments in airway epithelial cells [56]. CFBE41o- cells have been shown to express AMPK [57] and it is possible that pyocyanin caused an activation of AMPK thereby leading to an inhibition of CFTR activity.

Loss of total cellular GSH by pyocyanin

Reduced glutathione is the most abundant cellular thiol antioxidant and plays a critical role in the maintenance of the intracellular redox balance and redox-sensitive signaling events. A previous study showed that pyocyanin modulated the redox status in a complex manner [44]. By redox cycling with intracellular NADPH pyoycanin generated H_2O_2, which caused the formation of mixed disulfides by oxidation of protein and nonprotein thiol groups including GSH [44]. Since glutathione reductase must compete with pyocyanin for NADPH in order to reduce GSSG back to GSH, it became the rate limiting step that lowered intracellular levels of free sulfhydryl groups, including GSH. Our roGFP1 measurements showed that pyocyanin oxidized the cytosol by -48.0 ± 4.6 mV within 2-3 hours in CFTR-corrected CFBE41o- cells and a similar oxidation was reported in CF15 cells [48] suggesting that CFTR expression did not alter pyocyanin-induced oxidation of the cytosol. To complement these measurements we quantified the reduced and oxidized form of glutathione in cell lysates of CFTR-corrected CFBE41o- monolayers. Exposure to pyocyanin caused a significant decline of total cellular GSH to 62% of untreated monolayers within 24 hours. A similar value was reported in A549 lung epithelial cells and 16HBE14o- bronchial epithelial cells where total cellular GSH declined to 60% of untreated controls within 24 hours [45], and the cellular loss was accounted for by an efflux of GSH into the media [45]. Effects of pyocyanin on intracellular GSH levels have been detected as early as 30 min after exposure to pyocyanin [44]. We found that pyocyanin did not lead to a detectable increase in cellular GSSG in CFTR-corrected CFBE41o- cells, and a similar finding was previously reported in A549 lung epithelial cells [45] and HUVEC human endothelial cells, whereas 16HBE14o- bronchial epithelial cells showed a detectable increase in GSSG [45].

There are several possibilities that might have caused the loss of cellular GSH including the formation of H_2O_2 and mixed disulfides [44], a direct reaction between pyocyanin with cellular GSH (Fig. 1E and [45]), increased efflux of GSH from airway epithelial cells [45], as well as GSH conjugation [58], however no evidence was found for the conjugation of GSH with either the oxidized or reduced pyoycanin under cell-free conditions [44]. It is currently not known if pyocyanin decreases GSH by affecting the activity of enzymes involved in GSH synthesis.

The depletion of GSH by pyocyanin is of concern in chronic pseudomonal lung disease and in particular in patients with cystic fibrosis who are characterized by a systemic deficiency of GSH in the epithelial lining fluid in the lung [59]. The airways of most patients with CF become chronically infected with *P. aeruginosa*, and pyocyanin levels as high as ~130 μM have been measured in the sputum. A number of reports showed that pulmonary GSH levels are decreased in CF patients [59,60] and several studies reported alterations in glutathione transport which were associated with the ΔF508 mutation of the CFTR chloride channel [16,61]. Based on these reports we have previously measured the intracellular redox potential in CF and CFTR-corrected nasal epithelial cells and found that the cellular redox potential was not affected by CFTR expression. Based on these findings we consider that the presence of *P. aeruginosa*-derived pyocyanin could be the primary source that leads to oxidative stress and impairments in GSH status in the airways of CF patients *in vivo*.

Our transepithelial experiments showed that a decline in cellular GSH by 40% was associated with an inactivation of the cAMP-dependent CFTR Cl^- transport by 86% in CFTR-corrected CF bronchial epithelial cells. These data suggest that intracellular GSH levels may be important for the proper function of the cAMP-dependent CFTR Cl^- transport. CFTR possesses 18 cysteine residues that are possible targets for oxidation, and several oxidized forms of glutathione have been shown to glutathionylate and inhibit the CFTR channel [22]. In addition, the depletion of intracellular GSH was paralleled by a fall in intracellular ATP levels to 46% of untreated controls. It is well known that ATP is important for the proper gating of the CFTR

channel, and therefore loss of cellular ATP might present a factor for the inhibition of CFTR activity by pyocyanin.

In conclusion, this study demonstrated that pathophysiological concentrations of pyocyanin adversely affect bronchial epithelial cell redox status and CFTR function indicating that the salt and water transport by the airway epithelium and, as a result, the composition of the airway surface liquid will be affected by the presence of pyocyanin in *Pseudomonas aeruginosa*-infected lungs. The major effect of pyocyanin, i.e., the substantial inhibition of active CFTR Cl^- transport (Fig. 5D), is a critical component that will contribute to the impairments in epithelial fluid secretion and mucociliary clearance, and thus support the colonization of *P. aeruginosa*. In the CF lung, pyocyanin is a critical factor that needs to be considered for the outcome of clinical gene and drug therapy studies that are aimed at restoring CFTR function in the airway epithelium. The presence of high levels of pyocyanin in infected CF lungs may interfere with functional outcome measures for CFTR activity and hamper CF treatments.

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LIST OF ABBREVIATIONS

cAMP-I_{Cl}
: forskolin-stimulated chloride current

CF
: cystic fibrosis

CFTR
: cystic fibrosis transmembrane conductance regulator

CFBE41o-
: cystic fibrosis bronchial epithelial cell line

DPI
: diphenyl iodinium

GSH
: reduced glutathione

GSSG
: glutathione disulfide

I_{Cl}
: transepithelial chloride current

NADPH
: nicotinamide adenine dinucleotide phosphate

roGFP1
: redox sensitive green fluorescent protein mutant

ROS
: reactive oxygen species

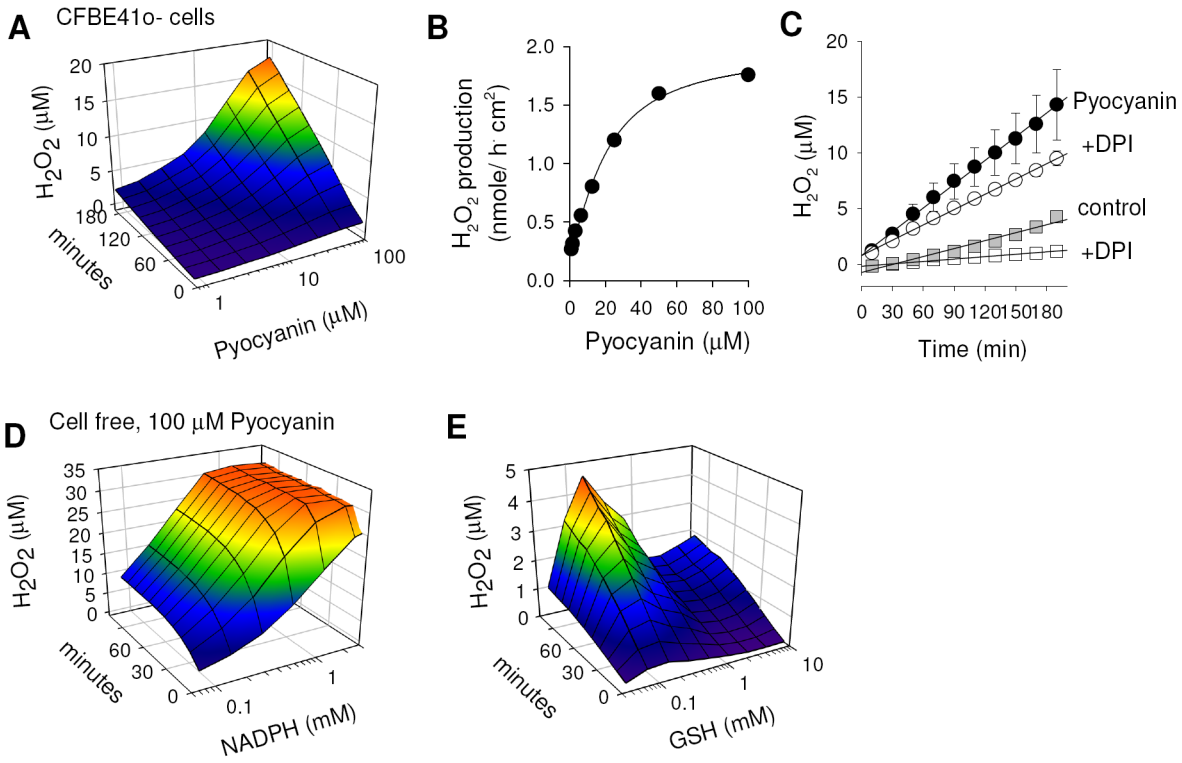

Figure 1. Pyocyanin generates H_2O_2 in bronchial epithelial cells

Production of H_2O_2 by pyocyanin was measured in CFBE41o- cells (A-C) or in cell-free experiments (D&E) using Amplex Red. (A) Time- and concentration-dependency of H_2O_2 release of CFBE41o- cells incubated with pyocyanin. Measurements were done with ~300,000 cells in 100 μl glucose-supplemented PBS. (B) Dose-dependency of pyocyanin-induced rates of H_2O_2 production (expressed as per 1 cm^2 epithelial area). Line represents fit of data to Hill-Michaelis-Menten function [42]. Maximal rate of H_2O_2 production was 1.67 ± 0.11 nmole·h^{-1}·cm^{-2}, with a halfmaximal pyocyanin concentration of 21.1 ± 2.0 μM, a Hill coefficient of 1.36 ± 0.15, and a pseudo first-order rate of ~52 pmole·h^{-1}·cm^{-2} per μM pyocyanin concentration; n = 8. (C) Comparison of H_2O_2 released from CFBE41o- cells in presence and absence of pyocyanin (100 μM) ± DPI (1 μM). Resulting rates (estimated by linear regression) were 0.45 ± 0.029 nmole·h^{-1}·cm^{-2} (untreated, n=25), 0.14 ± 0.006 nmole·h^{-1}·cm^{-2} (untreated + 1 μM DPI), 1.35 ± 0.025 nmole·h^{-1}·cm^{-2} (pyocyanin, n=3), and 0.87 ± 0.015 nmole·h^{-1}·cm^{-2} (pyocyanin + 1 μM DPI). (D&E) Time and concentration dependency of H_2O_2 production by NADPH (D) and GSH (E) in presence of pyocyanin (100 μM). Note different y axis scaling in D and E. A color gradient was added to panels A,D&E to visualize the H_2O_2 concentration in these plots.

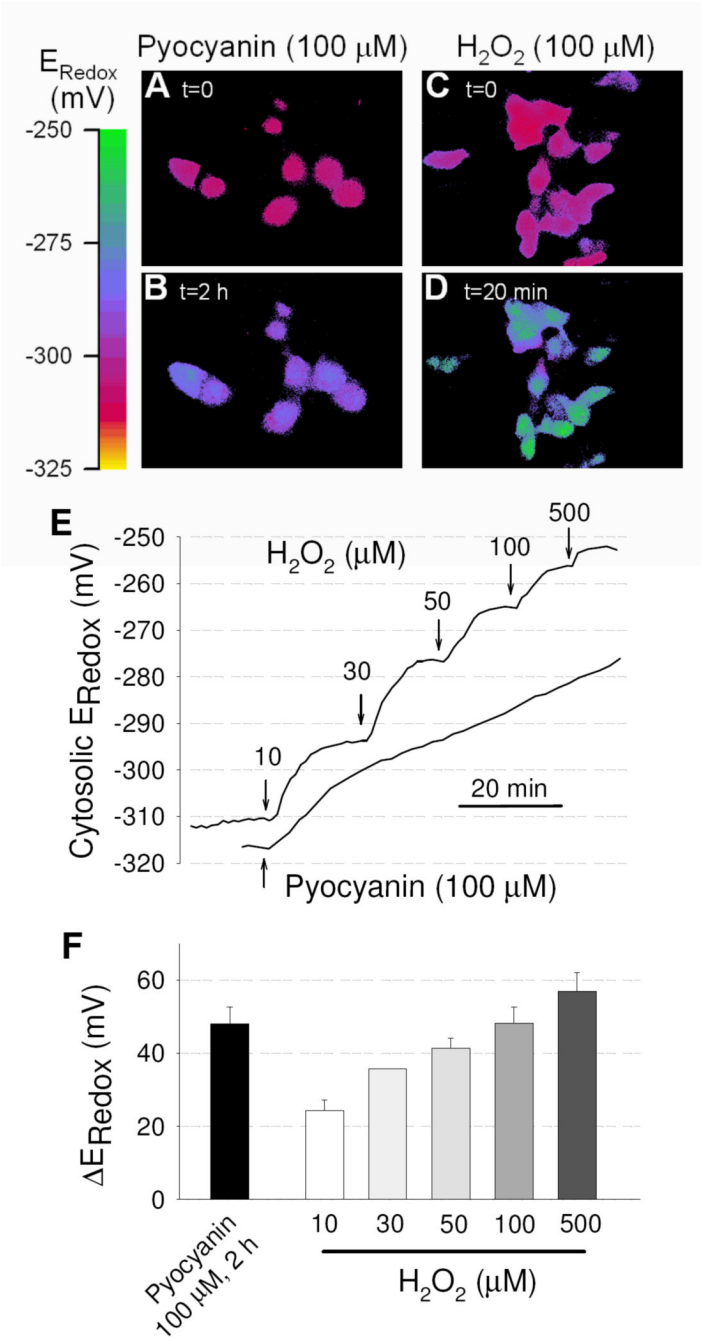

Figure 2. Oxidation of the cytosol of human bronchial epithelial cells by pyocyanin and H_2O_2

Cytosolic redox potential (E_{redox}) was measured in CFTR-CFB41o- cells by recombinantly expressing redox-sensitive roGFP1. (A-D) Pseudo-colored fluorescence ratio images of roGFP1-expressing cells during treatment with pyocyanin (A,B) or H_2O_2. (C,D) Colors correspond to the scale of redox potentials as shown by the scale bar. (E) Time-course of oxidation of the cytosol by pyoycanin (100 μM) in comparison to stepwise addition of 10-500 μM H_2O_2. Line represents average E_{redox} of >10 cells. (F) Average change in E_{redox} measured at steady state. Oxidation by 100 μM pyocyanin reached stable values after 2 hr treatment (*black bar*) and is compared to effects of increasing doses of H_2O_2 (*open bars*). Mean ± SE

for 1-3 determinations at each concentration. Measurements were done with ~100,000 cells in 100-500 μl saline solution.

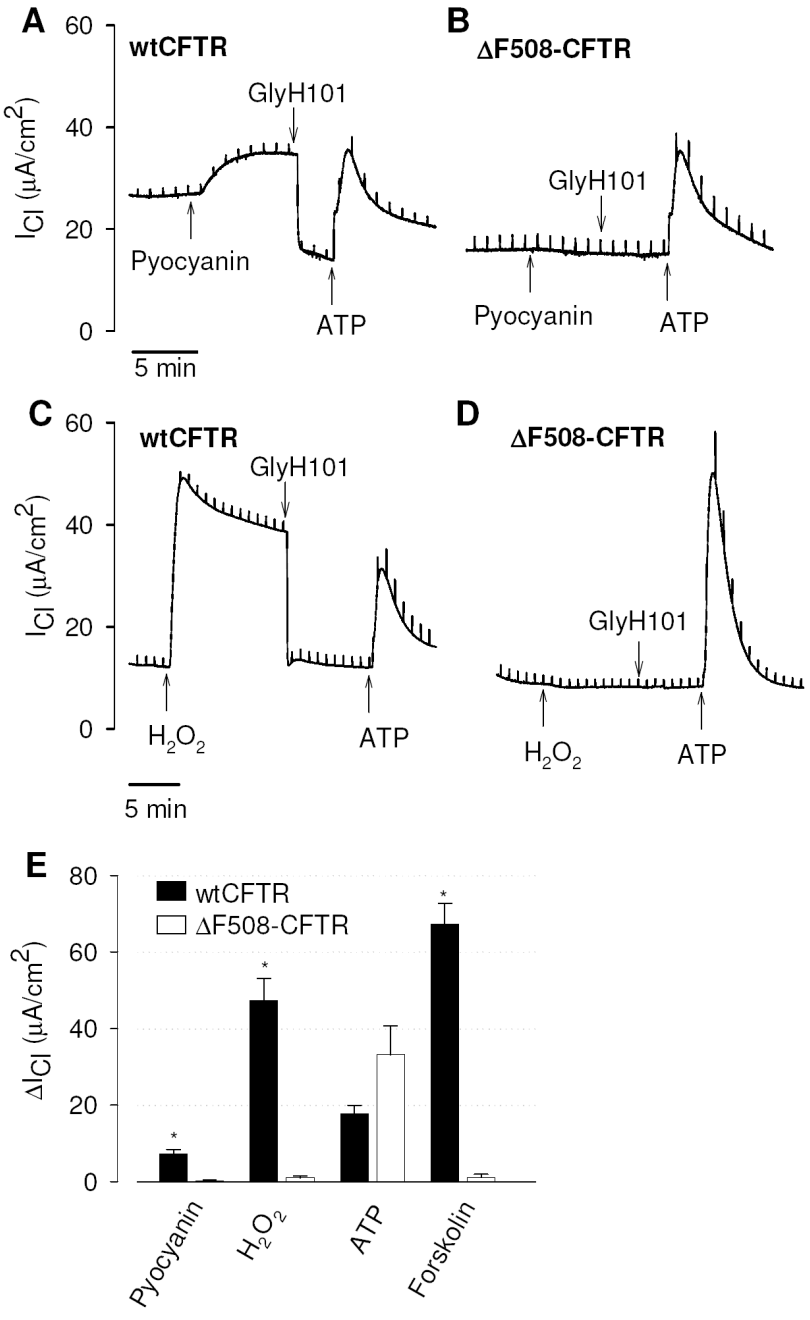

Figure 3. Activation of resting CFTR Cl^- currents by pyocyanin and H_2O_2 in CFTR-corrected but not CF human bronchial epithelial cells (CFBE41o-)

(A) Activation of Cl^- currents (I_{Cl}) by pyocyanin (100 μM) in CFTR-corrected CF monolayers (wtCFTR) and inhibition of pyoycanin-stimulated I_{Cl} by the CFTR blocker GlyH101 (20 μM). (B) Pyocyanin had no effect on I_{Cl} in CF monolayers homozygous for ΔF508-CFTR (ΔF508-CFTR) whereas ATP elicited a chloride secretory response similar to wtCFTR-expressing cells. (C,D) Corresponding activation of resting I_{Cl} by H_2O_2 (100 μM) in CFTR corrected but not CF monolayers, and inhibition of H_2O_2-stimulated I_{Cl} by the CFTR blocker GlyH101 (20 μM). Note that both oxidants stimulated the CFTR-mediated but not the calcium-activated Cl^- conductance. (E) Summary of stimulatory effects of pyocyanin (100 μM), H_2O_2 (100 μM),

ATP (500 μM), and forskolin (20 μM) on I_{Cl}. Data are mean values ± SE (n = 8-34 experiments), * denotes significantly different from wtCFTR, $p<0.05$. Measurements were done with ~1 Mio cells in 5 ml saline solution.

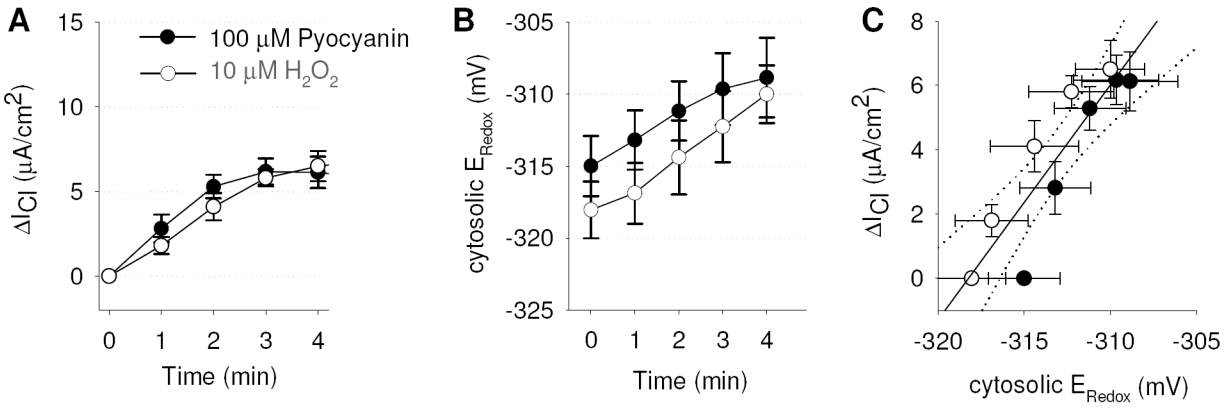

Figure 4. Short-term activation of CFTR Cl^- transport and corresponding cytosolic redox potential changes by pyocyanin

(A,B) Stimulation of Cl^- currents (ΔI_{Cl}) and intracellular redox potentials (E_{redox}) by 100 μM pyocyanin or 10 μM H2O2 in CFTR-corrected CFBE41o- monolayers plotted at 0, 1, 2, 3 and 4 minutes. Note that pyocyanin-induced ΔI_{Cl} and E_{Redox} were similar to those elicited by 10 μM H_2O_2. (C) Relationship between ΔI_{Cl} and E_{redox}; *line* is regression line with slope = 1.41 ±0.05 $\mu A \cdot cm^{-2} \cdot mV^{-1}$, offset = 446±13.6 $\mu A/cm^2$; *dotted line* is 95% confidence interval. Legend in panel A applies to all panels.

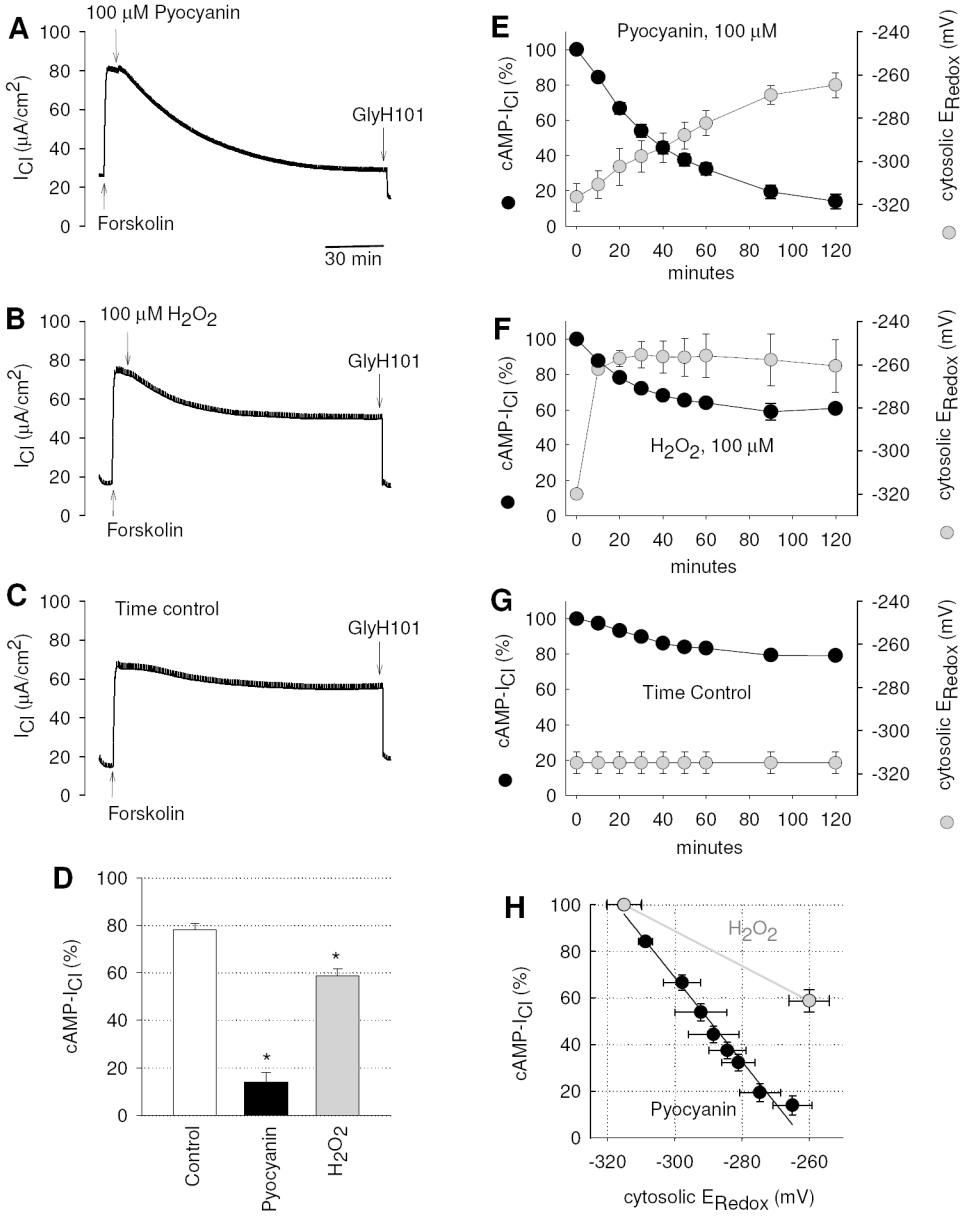

Figure 5. Long-term inhibition of forskolin-stimulated Cl^- transport and corresponding cytosolic redox potential changes by pyocyanin

Cl^- current (I_{Cl}) was activated by the cAMP agonist forskolin (20 μM) across CFTR-corrected CFBE41o- monolayers, and subsequent exposure to mucosal (A) Pyocyanin (100 μM) or (B) H_2O_2 (100 μM) inhibited I_{Cl}. (C) Untreated time control experiment. (D) Relative amounts of cAMP-dependent Cl^-currents (cAMP-I_{Cl}) measured 120 mins after addition of forskolin in time controls, and after treatment with pyocyanin or H_2O_2, * significantly different from control (ANOVA, $p<0.001$). (E) Pyocyanin-induced inhibition of forskolin-stimulated I_{Cl} (cAMP-I_{Cl}) occurs over the course of 2 hours and is paralleled by a gradual oxidation of the cytosol (E_{Redox}) at an average rate of 0.57 mV/min. (F) H_2O_2-induced inhibition of cAMP-I_{Cl} levels off after 60 min and rapidly oxidizes E_{Redox} at a rate of 5.9 mV/min. (G) Untreated time controls. (H) Relationship between inhibition of cAMP-I_{Cl} and time-matched

corresponding E_{Redox} for treatment with pyocyanin. For H_2O_2, data are plotted at time = 0 and after 120 min. Slopes were significantly different, H_2O_2, 0.75±0.07 %/mV, and pyocyanin, 1.81±0.1 %/mV ($p<0.001$, t test).

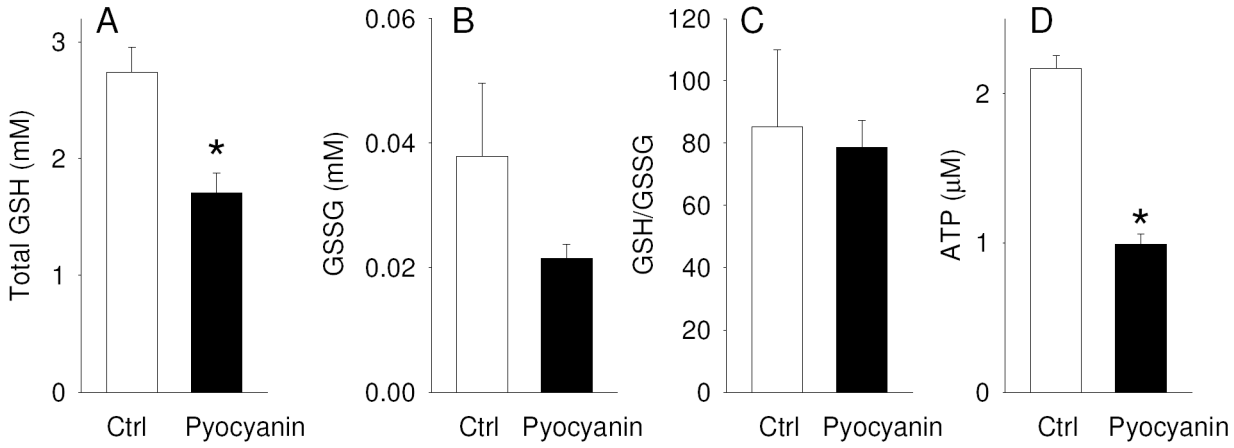

Figure 6. Effect of pyocyanin on GSH, GSSG, and ATP levels in CFBE41o- cells

CFTR-corrected CFBE41o- monolayers grown on cell culture inserts (area: 1 cm^2) were incubated with 1 ml cell culture medium in the absence (ctrl) or presence of 100 μM pyocyanin from the apical side for 24 h. (A) Total cellular GSH, (B) GSSG and (C) GSH/GSSG ratio were determined. Results shown are mean±SE, n=3. (D) Cells were grown in a 96-well plate (0.316 cm^2) and incubated with 100 μl cell culture medium in the absence or presence of 100 μM pyocyanin for 24 h, following which intracellular ATP levels were determined (n=8). *, significantly different from control, t-test. Measured concentrations are reported for the lysate (~50,000 cell in 100 μl).