Redox-independent Activation of NF-κB by Pseudomonas aeruginosa Pyocyanin in a Cystic Fibrosis Airway Epithelial Cell Line*

The roles of the Pseudomonas aeruginosa-derived pigment pyocyanin (PYO) as an oxidant and activator of the proinflammatory transcription factor NF-κB were tested in a cystic fibrosis (CF) airway epithelial cell line, CF15. 100 μM PYO on its own had no effect or only small effects to activate NF-κB (<1.5-fold), but PYO synergized with the TLR5 agonist flagellin. Flagellin activated NF-κB 4–20-fold, and PYO increased these activations >2.5-fold. PYO could have synergized with flagellin to activate NF-κB by redox cycling with NADPH, generating superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (HO$^·$). Cytosol-targeted, redox-sensitive roGFP1 and imaging microscopy showed that 1–100 μM PYO oxidized CF15 cytosol redox potential (Ψ$_{cyto}$) from −325 mV (control) to −285 mV. O$_2^-$ (derived from KO$_2^·$ or xanthine + xanthine oxidase) or H$_2$O$_2$ oxidized Ψ$_{cyto}$ dose-dependently but did not activate NF-κB, even in the presence of flagellin, and 400 μM H$_2$O$_2$ inhibited NF-κB. Overexpressing intracellular catalase decreased effects of PYO and H$_2$O$_2$ on Ψ$_{cyto}$ but did not affect flagellin + PYO-activated NF-κB. Catalase also reversed the inhibitory effects of H$_2$O$_2$ on NF-κB. The HO$^·$ scavenger DMSO did not alter the effects of PYO on Ψ$_{cyto}$ and NF-κB. The synergistic NF-κB activation was calcium-independent. Thus, in the presence of flagellin, PYO activated NF-κB through a redox- and calcium-independent effect.

Pseudomonas aeruginosa is commonly present in lungs of cystic fibrosis (CF) and immunocompromised patients (1, 2). The bacterium secretes a large number of products that contribute to virulence. These include type III-secreted exotoxins that disrupt the cytoskeleton and lyse the cells, as well as proteases, phospholipase, rhamnolipids, and hemolysin (3–8). In addition, P. aeruginosa produce and secrete the blue pigment pyocyanin (PYO), which is found in the sputum of patients with CF and bronchiectasis at concentrations up to 100 μM; this is responsible for the blue-green color often observed in CF sputum (4, 5, 9, 10). PYO-deficient P. aeruginosa elicits less mortality in P. aeruginosa-mediated burn-sepsis model in mice, and PYO appears to be important for persistence of P. aeruginosa in lungs of CF patients (3, 4, 11). PYO has a multitude of effects on the physiology of epithelial cells, including inhibition or alteration of antioxidant enzymes (12, 13), ciliary function (14), cellular metabolism, and organelle H$^+$-v-ATPase (2, 15). A key aspect of PYO pathology may result from its ability to trigger inflammation leading to the influx of neutrophils to the P. aeruginosa-infected region; PYO stimulates ICAM-1 and IL8 production on its own and also synergizes with IL1β and TNFα in stimulating IL8 production (16–18). The resulting IL8 production triggers polymorphonuclear leukocyte infiltration. The polymorphonuclear leukocytes are critical for fighting infections through production of reactive oxygen species (ROS) and proteases, but these products also contribute to the tissue destruction characteristic of CF.

PYO activation of IL8 production may occur through effects on cellular signaling leading to activation of the transcription factors AP-1, NF-IL6/C-EBP, and/or NF-κB, which control IL8 production (19). It is widely assumed that the effects of PYO on signaling are mediated at least in part through its ability to redox cycle with cellular NADPH and/or GSH leading to the production of ROS (9, 13, 20, 21) and oxidation of the cytosol and/or mitochondria (21, 22). Experiments using the electron spin resonance method showed that PYO caused the production of superoxide (O$_2^-$) but not hydroxyl radical (HO$^·$), indicating that O$_2^-$ and, through the action of superoxide dismutase, H$_2$O$_2$ might contribute to proinflammatory signaling by PYO (16). In addition, the antioxidant N-acetylcycteine and the HO$^·$ scavenger DMSO reduced the proinflammatory effects of PYO, consistent with PYO triggering inflammatory processes through its pro-oxidant effects (18). However, none of these experiments made direct, quantitative measurements on the proposed effect of PYO to produce ROS and oxidize the cytosol. In addition, there were no direct tests of the role of this hypothesized oxidation on the activation of inflammatory signaling in airway cells. Furthermore, because PYO synergizes with IL1β in triggering IL8 production and IL1β and flagellin trigger similar signaling (23), it was predicted that PYO would similarly syner-
gize with *P. aeruginosa* flagellin in activation of inflammatory signaling and production of IL8.

The present experiments were therefore designed to test the hypothesis that PYO activates inflammatory signaling by triggering production of the reactive oxygen species O$_2^\cdot$, H$_2$O$_2$, and HO$^\cdot$ that then oxidize the cytosol and activate NF-$\kappa$B. The experiments also tested whether this potentially proinflammatory effect was synergized in the presence of flagellin, the key product required for *P. aeruginosa* activation of inflammatory responses in airway epithelial cells (24, 25). The general approach was to measure redox potentials in the cytosol ($\Psi_{cyt}$) of the CF nasal cell line CF15 using genetically targeted, redox-sensitive GFP (roGFP1) (26–28) and ratiometric imaging microscopy during treatment with PYO, H$_2$O$_2$, and O$_2^\cdot$ to compare changes in redox potential to NF-$\kappa$B activation measured under the same conditions using both Western blot analyses of NF-$\kappa$B (p65), IKK, and IκB, and NF-$\kappa$B promoter-driven luciferase assays. The role of H$_2$O$_2$ in these processes was tested by overexpressing catalase (catalyzes hydrolysis of H$_2$O$_2$) and the role of HO$^\cdot$ by adding the HO$^\cdot$ scavenger DMSO. Finally, because PYO activation of NF-$\kappa$B appeared to be independent of redox changes and previous experiments showed a potential role for cytosolic [Ca$^{2+}$], we tested whether increases in cytosolic [Ca$^{2+}$] were involved.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Unless otherwise specified, reagents and chemicals were obtained from Sigma. Thapsigargin was dissolved in DMSO at 1.0 mM and then dissolved into solutions at 1–10 μM; these concentrations yielded similar effects on cellular functions.

**Pyocyanin**—PYO was purchased from Color Your Enzyme (Bath, Ontario, Canada). PYO was dissolved in PBS, pH 7.4, at 10 μM and diluted into medium or Ringer’s solution as mentioned in the text. To ensure complete solubility, we also dissolved PYO in DMSO, which was then added to the cells at the appropriate concentrations. Total [DMSO] in these experiments was 0.5%, which did not affect cellular responses. We observed no differences in responses to PYO that had been dissolved initially in PBS, Ringer’s, or DMSO.

**Flagellin**—*P. aeruginosa* flagellin (10$^{-3}$ g/ml in PBS, pH 7.4; Inotek, Beverly, MA) was stored at −20 °C and diluted from the stock into the incubation media at concentrations stated in the text. This solution was vortexed vigorously and heated to 37 °C before adding to the solutions to ensure dispersal as monomers. As described by Inotek, recombinant flagellin is expressed with tags in *Escherichia coli* and purified to >95% homogeneity by SDS-PAGE. Previous experiments showed that lipopolysaccharide contamination of this preparation is small and cannot account for effects of flagellin to activate NF-$\kappa$B (29). Flagellin isolated from *Salmonella typhimurium* (InvivoGen, San Diego) gave similar results. Flagellin was sensitive to freeze-thaw cycles, so comparisons among different treatment regimes were always performed with one batch of flagellin.

**Solutions**—In experiments to measure cytosolic redox potentials and Ca$^{2+}$, epithelial cells were incubated in Ringer’s solutions containing (in mM) the following: 145 NaCl, 1.2 MgSO$_4$, 2 CaCl$_2$, 2.4 K$_2$HPO$_4$, 0.6 KH$_2$PO$_4$, 10 HEPES, and 10 glucose, pH 7.4.

**Tissue Culture**—Cystic fibrosis airway cells JME/CF15, termed CF15 throughout this paper (30), a continuous SV40 large T antigen-transformed human nasal epithelial cell line homozygous for ΔF508 cystic fibrosis transmembrane regulator, were cultured in Dulbecco’s modified Eagle’s medium/F-12 media supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 200 milliunits/ml penicillin, 200 μg/ml streptomycin, 10 ng/ml epidermal growth factor, 1 μM hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 30 mM triiodothyronine, 180 μM adenine, and 5.5 μM epinephrine. Cells were passaged at a 1:5–1:10 dilution, and the remaining cell suspension was seeded directly onto 18-mm diameter cover glasses or onto 48-well or 24-well tissue culture plates (BD Biosciences).

**Measurement of Ca$^2+$**—High concentrations of PYO have been shown to elicit small increases in cytosolic [Ca$^{2+}$], Ca$_i$, through effects to release Ca$^{2+}$ from internal stores (presumably the endoplasmic reticulum) (20). Because increases in Ca$_i$ potentiate synergize with flagellin in stimulating NF-$\kappa$B in airway epithelial cells (19), this Ca$_i$-stimulating effect of PYO could be particularly important when the bacteria also produce and release flagellin into the airway surface liquid. We therefore tested whether PYO or H$_2$O$_2$ triggered changes in Ca$_i$ in CF15 cells. Cells grown on cover glasses were incubated with growth media containing 2 μM fura-2/AM for 40–60 min at room temperature and then washed three times with Ringer’s solution to remove the extra dye. Fura-2-loaded cells were mounted onto a chamber on the stage of the imaging microscope and maintained at room temperature. Treatments with agonists were made by diluting stock solutions into Ringer’s solution at the concentrations stated in the text. Fluorescence ratio imaging measurements of cytosolic Ca$_i$ were performed using methods that have been reported previously (19, 31). Briefly, a Nikon Diaphot inverted microscope was used with a 40× Neofluor objective (1.4 NA). A CCD camera collected emission (>510 nm) images during alternate excitation at 350 ± 5 and 380 ± 5 nm using a filter wheel (Lambda-10, Sutter Instruments, Novato, CA). Axon Imaging Workbench 4.0 (Axon Instruments, Foster City, CA) controlled both filters and collection of data. Calibration of fura-2 signals was performed as described by Grynkiewicz et al. (32). All images were corrected for background (region without cells).

**Confocal Microscopy**—Expression of roGFP1 in transiently transfected CF15 cells was analyzed on a Solamere spinning disk confocal microscope with excitation at 488 nm. Cells were bathed in Ringer’s solution containing 500 μM DTT to increase roGFP1 fluorescence intensity for excitation at 488 nm. Images were obtained using a 515 nm long pass emission filter and ×40 objective. Differential interference contrast images were also recorded to correlate cell morphology and roGFP1 fluorescence. Images were overlaid using Adobe Photoshop.

**Redox Potential Measurements Using roGFP1 and Imaging Microscopy**—Measurements of cytosolic redox potentials in CF15 cells were performed as described recently (28). Briefly, CF15 cells grown on coverslips were transiently transfected with plasmids coding for a redox-sensitive GFP mutant roGFP1 (26, 27) using the Effectene transfection reagent according to...

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the manufacturer’s protocol (Qiagen, Valencia, CA). roGFP1-expressing cells were bathed in Ringer’s solution and mounted in a chamber on the stage of a Nikon Diaphot microscope with a ×40 Neofluor objective (1.4 NA). Ratimetric imaging was performed using a CCD camera, filter wheel (Lambda-10, Sutter Instruments, Novato, CA), and Axon Imaging Workbench 4 (Axon Instruments, Foster City, CA) to collect emission (>510 nm) images during alternate excitation at 385 ± 5 and 474 ± 5 nm. Cells were exposed to the various treatments in Ringer’s solution, and roGFP1 ratios were recorded over time. Alternatively, ratios from multiple regions each containing ≥10 cells were recorded at the beginning of experiments and after 150 min of incubation with Ringer’s solution, 100 μM PYO, or 400 μM H₂O₂. At this time the ratios reached a steady state, i.e. no further change in roGFP1 ratio.

Calibration of the roGFP1 ratios in terms of cytosolic redox potentials was performed using a protocol that has been described previously (27, 28). Briefly, at the end of each experiment roGFP1 385:474 ratios were recorded during maximal oxidation by treatment with 10 mM H₂O₂ and then during maximal reduction by treatment with 10 mM DTT. Images were background-subtracted, and normalized roGFP1 385:474 ratios were averaged and converted to redox potentials (mV) using an in situ calibration curve that has been published previously (28). The calibration curve was generated by first preparing the standard solutions consisting of trans-4,5-dihydroxy-1,2-dithiane and DTT under nitrogen atmosphere to exclude oxidation by air. Then roGFP1-expressing cells were permeabilized by adding 1–5 μg/ml digitonin for 5–10 min. The permeabilized cells were then perfused with different DTT standard solutions covering redox potentials from −330 to −195 mV at pH 7 (calculated using the Nernst equation (27)), and 385:474 nm excitation ratios were recorded. The roGFP1 excitation ratios were normalized to the values measured using 10 mM DTTred as 0% oxidation and 10 mM H₂O₂ as 100% oxidation, and the normalized ratios were plotted against the calculated redox potentials of the DTT standard solutions to generate the curve (28) that was used for calibrating the experiments.

Phosphorylation of NF-κB p65, IKBa, and IKK—Immunoblot analysis was used to assay activity of NF-κB p65, IKBa, and IKKa/β. Cells were treated with pyocyanin, H₂O₂, and/or flagellin. Cells were treated with pyocyanin, H₂O₂, and/or flagellin for 20 or 40 min and then lysed in M-PER mammalian protein extraction reagent (Pierce) containing 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 50 mM calcium A. Protein concentrations were determined using Bradford reagent (Bio-Rad). Immunoblot analysis was performed by first separating protein (10–50 μg/lane) electrophoretically using SDS-PAGE and subsequently transferring to polyvinylidene difluoride membranes. Membranes were blocked (5% nonfat dried milk) in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 followed by incubation with specific antibodies. Primary antibodies (diluted 1:1000) for NF-κB p65, phospho-p65 (serine 536), IKBa, phospho-IKBa (serine 32), IKKa, and phospho-IKKa/β (serine 176/180) were purchased from Cell Signaling (Danvers, MA). Blots were first probed for phosphorylated proteins. The membranes were then stripped and probed with antibodies for nonphosphorylated proteins. Immunostaining of β-actin was performed to control for the amount of protein in each sample. Binding of primary antibodies was visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated secondary antibodies (1:2000) and Renaissance Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

Effects of Catalase on Redox and NF-κB—Overexpression of human catalase was obtained by overnight incubation of CF15 cells with an adenovirus coding for human catalase (100 multiplicities of infection). To control for adenoviral infection cells were infected with an adenovirus coding for β-galactosidase (100 multiplicities of infection). Cytosolic redox potentials were analyzed in CF15 cells after transfection with roGFP1-coding plasmids (see above), and transcriptional activation of NF-κB was studied by co-infection of cells with adv-catalase adv-NF-κB-luc in the presence of medium, [H₂O₂], or PYO with or without flagellin.

Statistics—Data have been presented as original values or as means ± S.D.; n refers to the number of averaged experiments. Significance was tested using t test for paired or unpaired samples as appropriate. Calculated p values <0.05 were considered significant.

RESULTS

Effects of PYO and Flagellin on NF-κB—Inflammatory signaling leading to the activation of NF-κB was assayed in CF15 airway epithelia by measuring phosphorylation/degradation of involved mediators in the presence or absence of flagellin (0.1 μg/ml, a submaximal dose; see Ref. 19) and PYO (100 μM, concentration found in sputum of CF patients; see Ref. 10). NF-κB...
Further experiments utilized NF-κB-regulated luciferase to test the effects of PYO and flagellin on NF-κB signaling. CF15 airway epithelia cells expressing the luciferase reporter gene driven by NF-κB transcriptional activation were exposed to increasing concentrations of PYO in the absence and presence of flagellin (0.1 μg/ml) (Fig. 1B). PYO on its own had a small stimulatory effect on NF-κB compared with untreated controls only at the highest concentration tested, 100 μM. Flagellin increased NF-κB-luciferase 5.5-fold over control, and this increase was further amplified when cells were treated with both flagellin + PYO. Physiologically relevant concentrations of PYO of 50 and 100 μM (as described for sputum of CF patients; see Ref. 10) synergized with flagellin in activating NF-κB 10- and 15.7-fold, respectively. Experiments were also performed to test the sidedness of these responses. Addition of flagellin and PYO to the apical side of cells grown to confluence on filter inserts yielded a synergistic activation of NF-κB with similar synergism compared with basal addition, although the magnitude of activation by flagellin and PYO was less when added to the basal side, perhaps because of restricted access to the membrane (Fig. 1C). Apical addition of flagellin and basal addition of PYO also yielded a synergistic activation of NF-κB (13.5-fold increase) compared with flagellin (5.5-fold) and PYO (1.2-fold). These results showed that flagellin and PYO synergized in activating NF-κB when they were exposed to either apical or basal side or even when flagellin and PYO were on opposite sides of the epithelium.

**Effects of PYO, H₂O₂, and O₂⁻ on NF-κB and Cytosolic Redox Potential**—As proposed previously (34), PYO is expected to react with cytosolic reducing agents like NADPH to generate O₂⁻ which would be expected to dismutate to H₂O₂ by cellular superoxide dismutase. Both H₂O₂ and O₂⁻ would be expected to oxidize the cytosolic redox potential. Expression of a redox-sensitive GFP mutant roGFP1 allowed the cytosol-specific analysis of the redox potential (Ψ_cytosol) in response to PYO. Typical images of roGFP1 expressed in CF15 cells showed that the sensor was localized throughout the cytosol and also in the nucleus (Fig. 2A). We detected no differences in the redox properties of the cytosol and nucleus of CF15 cells, consistent with previous results (26, 28). Changes in the 385:474 excitation ratio of roGFP1 fluorescence were converted to Ψ_cytosol (in mV) by applying a recently published calibration curve (28). As shown in Fig. 2B, PYO oxidized the cytosol of CF15 cells in a dose- and time-dependent way. 10 and 100 μM PYO both caused Ψ_cytosol to oxidize slowly over the course of 30–45 min to

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**FIGURE 1. Synergistic activation of NF-κB by PYO + flagellin in CF15 cells.** A, CF15 cells grown to confluency in wells were exposed to medium, 100 μM PYO, 10⁻² g/ml flagellin, or PYO + flagellin (Flag). Activation of IKKα/β, p65, and IκBα was analyzed after 20 and 40 min by observing protein phosphorylation and degradation using specific antibodies as indicated next to each panel. B, CF15 cells grown in wells were infected with NF-κB-luciferase adenovirus and then exposed to either apical or basal 100 μM flagellin or to apical flagellin and basal PYO for 4 h, followed by measurements of luciferase activity, expressed relative to controls (=1.0). Data are averages ± S.D. (n ≥ 5 experiments for each point). *, p < 0.05 compared with control; **, p < 0.05 compared with flagellin. C, CF15 cells grown to confluency on permeable filter inserts were infected with NF-κB-luciferase adenovirus and then exposed to either apical or basal 100 μM PYO + 10⁻² g/ml flagellin or to apical flagellin and basal PYO for 4 h, followed by measurements of luciferase activity, expressed relative to controls (=1.0). Data are averages ± S.D. (n ≥ 3 experiments for each point). *, p < 0.05 compared with control; **, p < 0.05 compared with flagellin. Effect of apical flagellin + basal PYO was compared with apical flagellin.
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![Image](60x316 to 288x733)

**FIGURE 2.** Expression of roGFP1 and PYO-induced oxidation of the cytosol and activation of NF-κB in CF15 cells. A, CF15 cells were transiently transfected with plasmids coding for cytosolic roGFP1. Confocal fluorescence image (excitation: 488 nm; emission >510 nm) overlaid on the bright field image was taken 48 h post-transfection on living cells in PBS containing 0.5 mM DTT to increase brightness at 488 nm. B, redox responses of cytosolic roGFP1 were measured during treatment of cells with 10 and 100 μM PYO in Ringer’s solution. Raw data were background-subtracted and calibrated into redox potentials. Experiment was typical of five similar experiments. C, summary of ΔΨcyto versus [PYO] measured in the steady state in experiments like that in B. Data are averages ± S.D. (n = 5 for each point). Cells that were also exposed to 10−7 g/ml flagellin (Flag) (closed circle) exhibited similar PYO-induced oxidation as cells that were not exposed to flagellin (open circles). *p < 0.05 compared with control.

new steady values. These effects of PYO were relatively irreversible, as ΔΨcyto remained oxidized even after 60 min following removal of PYO from the bathing solutions for 60 min. On average 10 and 100 μM PYO oxidized ΔΨcyto from −325 to −306 mV and −285 mV, respectively (Fig. 2C). Flagellin did not affect ΔΨcyto in the absence (data not shown) or presence of PYO (filled circle Fig. 2C).

If PYO were activating NF-κB through its effect to generate O₂⁻ and H₂O₂, it was expected that exogenous addition of these ROS would also oxidize the cytosol and activate NF-κB. Effects of O₂⁻ were investigated by treating CF15 cells with either xan-thine + xanthine oxidase or KO₂. Xanthine (X) alone or xanthine oxidase (XO) alone (not shown) had no effect on ΔΨcyto, but addition of both X + XO caused rapid oxidation of ΔΨcyto by up to 50 mV (Fig. 3A). These results were consistent with the idea that extracellular X + XO generated O₂⁻, which entered the cell and oxidized the cytosol. Similar oxidation occurred during addition of KO₂ (Fig. 3B). Although O₂⁻ (either from X + XO or from KO₂) effectively oxidized the cytosol, it did not activate NF-κB either on its own or when added with flagellin (Fig. 3, C and D).

Similarly, if PYO were activating NF-κB through its effect to generate H₂O₂, exogenous addition of H₂O₂ should oxidize the cytosol and activate NF-κB. H₂O₂ dose-dependently (threshold effect at 1 μM, highest concentration applied 400 μM) oxidized ΔΨcyto from −325 to −250 mV (Fig. 4, A and B). Although such single additions of H₂O₂ effectively oxidized the cytosol, H₂O₂ (added either alone or in combination with flagellin) did not affect NF-κB-luciferase activity (Fig. 5A).

Previous experiments (35) showed that H₂O₂ can be metabolized by cells and that repeated additions of H₂O₂ to the media bathing the cells were required to maintain extracellular concentrations. We therefore tested the possibility that the lack of effect of H₂O₂ on NF-κB was caused by transient oxidation of ΔΨcyto resulting from single additions of H₂O₂. As shown in Fig. 4C, 25 and 100 μM H₂O₂ both oxidized ΔΨcyto over the course of 15–20 min followed by a slow reduction of ΔΨcyto over time back toward control levels. In contrast, sequential additions of H₂O₂ caused ΔΨcyto to remain oxidized to a roughly constant level of −310 mV (10 μM H₂O₂), −290 mV (25 μM H₂O₂), and −270 mV (100 μM H₂O₂) (Fig. 4D).

Armed with this information on the time and concentration dependence of effects of H₂O₂ on redox potentials, we tested for effects on activation of NF-κB in CF15 cells. Although 0.1 μg/ml flagellin induced typical phosphorylation of IKKα/β, p65, and IκBα after 30 and 40 min (Fig. 1A), 100 μM H₂O₂ elicited no changes in phosphorylation of IKKα/β, p65, and IκBα in the absence or presence of 0.1 μg/ml flagellin (not shown). Further tests were performed using the sequential-addition protocol (Fig. 4D) to obtain relatively constant cellular oxidative redox potentials over the 4 h required for the NF-κB-luciferase experiments. Using this protocol, H₂O₂ had no effect (at 25, 50, and 100 μM) or only inhibitory effects (at 400 μM) on NF-κB activity when added alone or in the presence of flagellin (Fig. 5B, open circles). 1 and 10 μM H₂O₂ also had small oxidizing effect on ΔΨcyto (Fig. 4A), but there were no effects of these concentrations of H₂O₂ (added either alone or in the presence of flagellin) on NF-κB (not shown).

A second approach to testing a role for H₂O₂ in oxidizing ΔΨcyto and activating NF-κB was to use an adenovirus to over-express intracellular catalase (converts H₂O₂ to H₂O and O₂) in CF15 cells. β-Galactosidase (lacZ) adenovirus was used as a control. These cells were also infected with NF-κB-luciferase adenovirus to measure NF-κB activity or transfected with roGFP1 to measure ΔΨcyto lacZ-infected (Fig. 4E) and uninfected control cells (Fig. 4A) exhibited similar changes in ΔΨcyto in response to H₂O₂. In contrast, catalase-expressing cells exhibited much smaller oxidations of ΔΨcyto in response to H₂O₂.
Effects of PYO and NF-κB on NF-κB: Role for Ca$_{i}$?—Previous research showed 80–300 μM PYO triggered increases in Ca$_i$ in human bronchial epithelial cells and A549 cells (20) and that increases in Ca$_i$ synergized markedly with flagellin in activating NF-κB and IL8 secretion (19). We therefore tested whether the synergism noted between flagellin and PYO in activating NF-κB involved similar changes in Ca$_i$ in CF15 cells. As shown in Fig. 7, 100 μM of PYO had no effect on Ca$_i$ (initial Ca$_i$ 48 ± 12 nm versus Ca$_i$; after PYO treatment, 64 ± 11 nm), although a typical increase in Ca$_i$ was recorded upon stimulation with the known Ca$_i$-regulating agents ATP (activates purinergic receptors) and thapsigargin (blocks Ca$_{2+}$ pump in the endoplasmic reticulum, leading to loss of Ca$_{2+}$ into the cytosol) (see Ref. 19). Previous experiments also showed flagellin did not alter Ca$_i$ in CF15 cells (19). Therefore, the synergistic activation of NF-κB by PYO and flagellin in CF15 cells did not require changes in Ca$_i$.

DISCUSSION

PYO Synergizes with Flagellin in Activating NF-κB—A major conclusion from these studies was that during the initial stages of exposure (0.5–4 h) PYO elicited no activation or only small activation of NF-κB on its own, but large (>2.5-fold) synergistic stimulation when flagellin was also present. Unpublished experiments have shown similar synergism between PYO and TNFα in activating NF-κB-luciferase (data not shown). Previous experiments showed that 24 h of exposure to PYO elicited similar activation of NF-κB in the presence of TNFα or IL1β (18). Thus, PYO elicited synergistic activation of NF-κB in the presence of agonists that activated NF-κB. The synergy between flagellin and PYO occurred during additions to the apical or basolateral side of the monolayers and also when flagellin was added apically and PYO basally. It therefore seems likely that the synergistic interactions between PYO and flagellin occurred through interactions among cytosolic signaling pathways and not through interactions at the cell surface. Because NF-κB is a key regulator of IL8 production and secretion (19) and [PYO] in the spumt of CF patients can reach 100 µM (4, 10, 15), these and previous (4, 11) data show that PYO may be an important modulator of innate immune responses to P. aeruginosa in vivo. During chronic infections in CF, P. aeruginosa lose their flagella and become immotile, so the concentration of flagellin in the spumt will likely decrease. However, even in this condition PYO may be an important modulator of

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3 C. Schwarzer and T. E. Machen, unpublished observations.
that is likely also resulted from its rapid oxidation at concentrations that oxidized the cytosol, which changes in response to PYO or H$_2$O$_2$. (i) Endogenous generation of ROS and oxidation of cytosol, likely resulting from rapid penetration of nonpolar, membrane-permeant H$_2$O$_2$ through the membrane bilayer. The rapid oxidation of cytosol in response to increased extracellular O$_2^-$ likely also resulted from its rapid penetration across the membrane, cytosolic conversion to H$_2$O$_2$, and subsequent oxidation of cytosol.

Activation of NF-κB by PYO Occurs through a Nonredox-mediated Effect—Several observations indicated that the PYO-induced generation of ROS and oxidation of cytosol played only a minor role in the ability of PYO to synergize with flagellin in activating NF-κB during a 4-h exposure. (i) Exogenous additions of H$_2$O$_2$ or O$_2^-$ at concentrations that oxidized the cytosol similar to that exhibited by 100 μM PYO (i.e., ~40 mV, from control ~325 mV to about ~285 mV in the presence of PYO), H$_2$O$_2$, or O$_2^-$ had no effect on NF-κB alone or in the presence of flagellin. (ii) The oxidizing effect of PYO was reduced in cells overexpressing catalase, but there was no significant effect of catalase expression on the synergistic stimulation of NF-κB by PYO + flagellin. (iii) The HO$^-$ scavenger DMSO had no effect on PYO-induced oxidation of cytosol, or activation of NF-κB by flagellin + PYO.

In contrast to the present work showing PYO elicited its stimulatory effects on NF-κB through nonredox mechanisms, previous work (18) showed there were important roles for H$_2$O$_2$, HO$^-$, and nitric oxide in the activation of IL8 and ICAM production over 1–2 days of exposure to PYO and one of its precursors, phenazine 1-carboxylic acid. A possible explanation for the differences between these previous (18) and present results could be that the redox-dependent proinflammatory

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**FIGURE 4. Effects of H$_2$O$_2$ and catalase on cytosolic redox in CF15 cells.** A, $\Psi_{cyto}$ was measured during short term treatment of roGFP1-expressing cells with 1, 10, and 100 μM H$_2$O$_2$ in Ringer’s solution. Experiment is typical of 13 similar experiments. B, summary change in $\Psi_{cyto}$ (compared with controls) as function of [H$_2$O$_2$]. Average ± S.D. (n = 13 experiments); *, p < 0.05 compared with control. C, $\Psi_{cyto}$ was measured during 2 h of treatment with 25 or 100 μM H$_2$O$_2$. There was a rapid increase during the first 15 min followed by slower decrease toward control levels over the course of about 100 min. D, $\Psi_{cyto}$ was measured during repeated treatment of cells with 25 μM H$_2$O$_2$. Experiment is typical of three similar experiments. E, dose-dependent effectsubsequent oxidation of cytosol and generation of O$_2^-$ are shown. *, p < 0.05 compared with no catalase expression.
effects of PYO and phenazine 1-carboxylic acid on IL8 and ICAM1 may be mediated through signaling pathways that lead to other transcription factors besides NF-κB that are important for overall gene regulation, e.g., NF-IL6 and/or AP-1 in the case of the IL8 promoter (19). Another possibility is that the redox-dependent proinflammatory effects of PYO on IL8 and ICAM1 resulted from the longer time course of the previous experiments. Thus, the proinflamatory effects of PYO could result from nonredox activation of NF-κB during 4-h incubations but by generating cellular ROS that activate proinflammatory signaling during 1–2-day incubations.

Because PYO oxidation of \( \Psi_{\text{cyt}} \) appeared not to be involved in activating NF-κB, how does PYO work? Western analysis showed that PYO and flagellin each increased phosphorylation of IKK and p65, and there were increased phosphorylations in the presence of both PYO and flagellin. This may indicate that some cells during exposure to high [PYO], PYO synergism with flagellin in activation of NF-κB does not require increases in \( \text{C}_{\text{a}} \), in CF15 and Calu-3 cells.

We considered the possibility that PYO elicited its stimulatory effects by altering redox properties in other cellular...
compartments besides the cytosol. Altering the redox properties of the endoplasmic reticulum could trigger an unfolded protein response that leads to activation of NF-κB (37), but PYO did not activate the unfolded protein response (analyzed by IRE1α-dependent splicing of XBP-1), indicating that the threshold for the inhibitory effect of PYO-induced oxidation of Ψmito could contribute to activation of NF-κB. Further studies will be required to determine the role of mitochondrial redox in controlling proinflammatory signaling in the cytosol.

Too Much Oxidation Inhibits NF-κB—Flagellin-activated NF-κB was slightly inhibited by extracellular treatments with O2-, which oxidized Ψcyto from control ~325 mV to about −270 mV (Fig. 3), and NF-κB was nearly completely inhibited by 400 μM H2O2, which oxidized Ψcyto to about −260 mV (Fig. 4). That this inhibitory effect on NF-κB resulted directly from oxidizing effects of H2O2 was shown by the reversal of the inhibition by overexpressing catalase. In contrast, overexpressing catalase prevented PYO-induced oxidation of Ψcyto, but did not affect activation of NF-κB. Taken together, these results indicated that the threshold for the inhibitory effect of excessive oxidation of Ψcyto on NF-κB in CF15 cells may occur near −270 mV.

Controversial Role of Redox Regulation of Inflammation—Previous data have shown that cytosolic oxidation on its own or in combination with cytokines activates NF-κB (35, 39, 40) and that antioxidants may reduce proinflammatory signaling (18). In contrast, others have provided evidence showing that oxidation can mediate anti-inflammatory effects (41, 42). The present data showed that NF-κB activity was insensitive to oxidation of Ψcyto by up to 40–50 mV, but further oxidations past this threshold had large inhibitory effects. These contrasting results indicate that responses to oxidation are likely to depend on subtle, cell-specific effects that may alter the magnitudes and localizations of oxidation. Furthermore, our data as well as that of others (43, 44) emphasized that at least some redox-active molecules can alter proinflammatory NF-κB signaling through redox-independent mechanisms. Future quantitative investigations of the role of Ψcyto in controlling proinflammatory signaling may yield insights into why oxidants can either stimulate, inhibit, or have no effect on inflammatory signaling in different cells.

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