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Pyocyanin and Its Precursor Phenazine-1-Carboxylic Acid Increase IL-8 and Intercellular Adhesion Molecule-1 Expression in Human Airway Epithelial Cells by Oxidant-Dependent Mechanisms[1]

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Pseudomonas aeruginosa **secretes numerous factors that alter host cell function and may contribute to disease pathogenesis. Among recognized virulence factors is the redox-active phenazine pyocyanin. We have recently demonstrated that the precursor for pyocyanin, phenazine-1-carboxylic acid (PCA), increases oxidant formation and alters gene expression in human airway epithelial cells. We report in this work that PCA and pyocyanin increase expression of ICAM-1 both in vivo and in vitro. Moreover, phenazines enhanced cytokine-dependent increases in IL-8 and ICAM-1. Antioxidant intervention studies indicated both similarities and differences between PCA and pyocyanin. The thiol antioxidant *N*-acetyl cysteine, extracellular catalase, and inducible NO synthase inhibitors inhibited ICAM-1 and IL-8 increases in response to both phenazines. However, pyocyanin was significantly more sensitive to *N*-acetylcysteine inhibition. Interestingly, hydroxyl radical scavengers inhibited the response to pyocyanin, but not to PCA. These studies suggest that *P. aeruginosa* phenazines coordinately up-regulate chemokines (IL-8) and adhesion molecules (ICAM-1) by mechanisms that are, at least in part, oxidant dependent. However, results indicate that the mechanisms by which PCA and pyocyanin exert their effects are not identical, and not all antioxidant interventions are equally effective in inhibiting phenazine-mediated proinflammatory effects.** *The Journal of Immunology,* **2005, 175: 4017–4023.**

Pseudomonas aeruginosa is an opportunistic human pathogen that causes an acute lung disease with high mortality in seriously ill and immunocompromised patients (1–4). *P. aeruginosa* is also commonly associated with a chronic, progressive lung disease in individuals with cystic fibrosis (CF)[3] (5–7). In both acute and chronic infections, the bacterium often exists as biofilms (8, 9).

The mechanism(s) by which *P. aeruginosa* establishes infection in these patients, as well as the mechanisms that underlie the pathophysiology of the resulting disease, remain poorly understood. However, numerous studies provide evidence that factors secreted by the bacterium could contribute to disease pathogenesis (5). Among these factors is pyocyanin, a redox-active phenazine derivative that increases intracellular oxidant stress (10–12). Pyocyanin has been detected at concentrations as high as 100 μM in pulmonary secretions from patients with *P. aeruginosa*-associated airway disease (13), and its production is increased when the organism is in the biofilm form (14, 15). Moreover, phenazines have been shown to be virulence factors using *Caenorhabditis elegans* and murine models (16–19). Of particular note are recent studies indicating that phenazine-deficient mutants are less virulent than wild-type controls and that pyocyanin production appears to provide a growth or survival advantage in vivo (19).

Studies from our laboratory demonstrate that both laboratory and clinical strains of the bacterium also secrete phenazine-1-carboxylic acid (PCA), the precursor for pyocyanin, and that PCA increases oxidant production, IL-8 release, and ICAM-1 expression by human airway epithelial cells (20). Moreover, like pyocyanin, PCA inhibits cytokine-dependent expression of RANTES and MCP-1. Although PCA and pyocyanin share a number of biological effects, the structural differences between the two predict differences in redox chemistry and, hence, potential differences in their mechanisms of action.

Inflammation generally involves the coordinate up-regulation of chemoattractants and cellular adhesion molecules that mediate interactions between immune and nonimmune cells. Among the adhesion molecules up-regulated in the *P. aeruginosa*-infected airway is ICAM-1 (21, 22). To our knowledge, our studies were the first to examine the effect of *P. aeruginosa* phenazines on ICAM-1 expression (20). Additionally, relatively little is known about the molecular mechanisms by which PCA and pyocyanin exert their effects on host gene expression. The observation that pyocyanin-dependent increases in IL-8 are delayed contrasts with the effects of host cytokines (23). Moreover, pyocyanin synergizes with host cytokines in increasing IL-8. Together these data suggest that pyocyanin may exert

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[3] Abbreviations used in this paper: CF, cystic fibrosis; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMTU, dimethylthiourea; KC, keratinocyte chemoattractant; L-AME, L-arginine methyl ester; NAC, *N*-acetylcysteine; NHBE, normal human bronchial epithelial cell; PCA, phenazine-1-carboxylic acid; SOD, superoxide dismutase.

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its effects through mechanisms that are, at least in part, distinct from those of inflammatory cytokines.

Because a neutrophilic inflammatory response is believed to be the major cause of airway damage in *P. aeruginosa*-infected airways, it was of considerable interest to examine further the effect of *P. aeruginosa* phenazines on expression of proinflammatory molecules and to determine whether antioxidant interventions inhibited these effects.

Materials and Methods

Materials

N-acetylcysteine (NAC), cycloheximide, bovine erythrocyte cytosolic superoxide dismutase (SOD), bovine liver catalase, and *Aspergillus niger* catalase were purchased from Sigma-Aldrich. DMSO and dimethylthiourea (DMTU) were purchased from Fisher Scientific and Aldrich, respectively.

Phenazine purification

PCA and pyocyanin were purified from bacteria-conditioned medium, as previously described (20). Final phenazine concentrations were determined spectrophotometrically, and purity was verified by HPLC (20). Purification by this method renders preparations with no detectable *P. aeruginosa* autoinducer or LPS (23).

Murine studies

Animal studies were performed under a protocol approved by the University of Iowa Institutional Animal Care and Use Committee, as previously described (24–26). Six-week-old C57BL/6J mice (Taconic Farms) underwent orotracheal intubation with a 24-gauge i.v. catheter and immediate airway injection of 50 μl of HBSS or HBSS with the indicated concentration of PCA or pyocyanin, followed by 100 μl of air. After the indicated time, mice were anesthetized and then euthanized by cervical dislocation, the lungs were exposed, and the pulmonary vascular system was flushed via the right ventricle with sterile saline. Airway leukocytes were isolated by bronchoalveolar lavage of the left lung with 0.5-ml aliquots of PBS, followed by quantitation of total leukocyte counts using a hemocytometer and differential counts using Wright's staining of Cytospin preparations (26). The four lobes of the right lung were ligated at their roots and removed under sterile conditions. For chemokine quantitation, homogenates from two lobes were tested for mouse keratinocyte chemoattractant (KC) and MIP-2 levels using ELISA and matched Abs from R&D Systems; observed range for each chemokine was 15–2500 pg/ml. The remaining lobes were fixed and immunostained for ICAM-1, as previously described (25).

Cell culture

The human respiratory epithelial cell lines A549 and 16-HBE14o-, as well as a CF cell line IB3 and its rescue cell line C38 were also cultured, as previously described (23). Primary normal human bronchial epithelial cells (NHBE) and bronchial/tracheal epithelial cell growth medium were purchased from Cell Applications. Primary cells (passages 1–5) were cultured on human placental collagen-coated tissue cultureware in bronchial/tracheal epithelial cell growth medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Polarized monolayers of normal and CF airway epithelial cells were obtained from the Cystic Fibrosis Cell Culture Core Facility (University of Iowa).

Measurement of human IL-8

Medium from control and stimulus-treated human cells was assayed for IL-8 by ELISA using matched Abs from R&D Systems, as previously described (23). The standard curve was in the range of 15–1000 pg/ml.

Cell-based ELISA for ICAM-1

ICAM-1 was measured in airway epithelial cells using a cell-based ELISA, as previously described (20).

Inhibitor studies

Cells were grown in 48-well plates until confluent. Cells were pretreated with inhibitor for 1–2 h before addition of agonist, and incubations (36–48 h) were done in the continued presence of inhibitor. Cycloheximide was removed after 6 h of incubation to avoid cytotoxicity.

Cytotoxicity assay

To test for cytotoxicity by inhibitors, cells were treated with and without inhibitor for 24 and 48 h. Cultures were then washed twice with incubation buffer (see above). A solution of 10 μM calcein-AM in incubation buffer was then added, and increases in fluorescence (λex, λem; 485/538) over time (0–60 min) at 37°C were measured using the BMG Fluostar microplate fluorometer (BMG Labtechnologies). Only noncytotoxic inhibitor concentrations were used throughout.

Data analysis

Data are expressed as mean ± SEM. Experimental results under different conditions were compared by Student's *t* test when independent variables were being assessed and by ANOVA when analyses of trends were being determined (time courses; concentration dependence). For both types of analyses, significance was defined as $p < 0.05$.

Results

Phenazines are proinflammatory in vivo

Previous studies showed that pyocyanin stimulates a neutrophilic inflammatory response in animal models (19, 27). To determine whether PCA was also sufficient to elicit an inflammatory response in the murine airway, 50 μl of HBSS alone or with 50–100 μM PCA or pyocyanin was introduced into the mouse airway by orotracheal injection. In preliminary time course studies, we found that a single injection of PCA or pyocyanin stimulated a time-dependent increase in neutrophils in bronchoalveolar lavage fluid (data not shown). In subsequent studies, multiple injections were found to be optimal for measuring increases in chemokine and ICAM-1 expression. Under these conditions, there was an increase in the concentration of murine neutrophil chemokines KC and MIP-2 in lung homogenates from phenazine-treated animals (Fig. 1*A*) relative to controls that were below detectable limits. Values reached statistical significance ($p < 0.05$), except in the case of the

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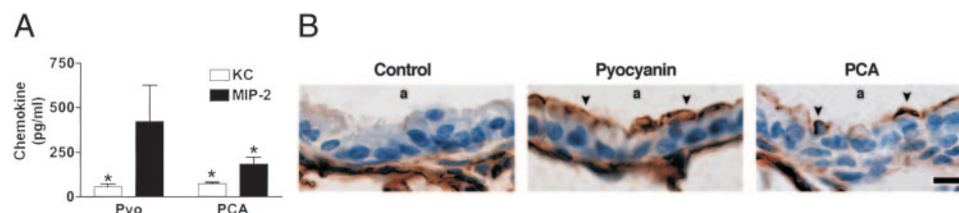

FIGURE 1. Proinflammatory effects of phenazines in the murine airway. *A*, Fifty microliters of HBSS or HBSS with 100 μM PCA or pyocyanin was introduced in each of three orotracheal injections over a 24-h period, and lungs were harvested 24 h after the last injection. A lung homogenate was prepared, and KC and MIP-2 were measured using ELISA. Readings for HBSS controls were below the level of detection for the assay (15 pg/ml). Data represent the mean ± SEM ($n = 3$); *, $p < 0.05$. Due to high variability, values for pyocyanin-treated animals at 48 h did not reach statistical significance, $p = 0.08$. *B*, Fifty microliters of HBSS or HBSS with 50 μM PCA or pyocyanin was introduced by orotracheal injection five times over a 36-h period, and lungs were harvested 6 h after the last injection. Lungs were fixed and sectioned for immunostaining with anti-ICAM-1. Representative images are shown for buffer controls and for phenazine-treated animals. Arrows indicate ICAM-1 staining at the apical (a) surface of the airway epithelium. Scale bar, 10 μm.

more variable 48-h samples from pyocyanin-treated animals (p = 0.08). Finally, increased ICAM-1 was observed at the apical surface of the airway epithelium in immunostained tissues from animals exposed to PCA or pyocyanin (Fig. 1*B*, arrows). These data indicate that PCA, like pyocyanin, is proinflammatory in the murine airway and demonstrate for the first time phenazine-dependent up-regulation of ICAM-1 in vivo.

Effect of phenazines on expression of ICAM-1

Based on these in vivo results and our preliminary findings that both pyocyanin and its precursor PCA increased release of IL-8 and surface expression of ICAM-1 by human airway epithelial cells (20), we designed additional experiments to identify potential mechanisms for these effects. As a first approach to address this question, A549 cells were treated with increasing concentrations of PCA or pyocyanin for 48 h and ICAM-1 surface expression was measured using a cell-based ELISA. Under these conditions, PCA and pyocyanin significantly increased ICAM-1 in a concentration-dependent manner (Fig. 2*A*). Moreover, as previously reported for pyocyanin, the PCA-dependent increase in IL-8 release was concentration dependent with maximal effects at ~50 μM (Fig. 2*B*): higher concentrations were cytotoxic to the cells. We next examined the time course for changes in ICAM-1 expression in A549 cells using 50 μM PCA or pyocyanin (Fig. 2*C*) and observed a time-dependent increase in response to each phenazine.

We earlier reported that pyocyanin-dependent increases in IL-8 showed a significant lag (~12–16 h) that contrasted with host cytokines such as TNF-α (23). Subsequently, we observed that the effects of PCA on IL-8 expression were significantly delayed (Fig. 2*D*). The basis for the delay in the response to phenazines is currently under study.

A549 cells have proven to be a good model for expression of proinflammatory cytokines by human airway epithelial cells. However, it was important to verify that findings in A549 cells reflected effects in other cell model systems. In this respect, we observed a significant increase in ICAM-1 in response to a 48-h treatment with 50 μM pyocyanin using the CF cell line IB3 (1.4 ± 0.06-fold control, mean ± SEM, n = 9, $p < 0.001$) and its rescue cell line C38 (1.9 ± 0.39, n = 10, $p < 0.05$). Moreover, 20 μM PCA or pyocyanin increased ICAM-1 and IL-8 expression in primary cultures of NHBE from Cell Applications (data not shown).

Of note, basal levels of ICAM-1 in the human epithelial cell lines 16-HBE14o-, Calu-3, and BEAS-2B, as well as in polarized monolayers of primary human bronchial epithelial cells grown using the air-liquid interface method, were relatively high (data not shown). This high basal expression made it difficult to detect potential increases in ICAM-1.

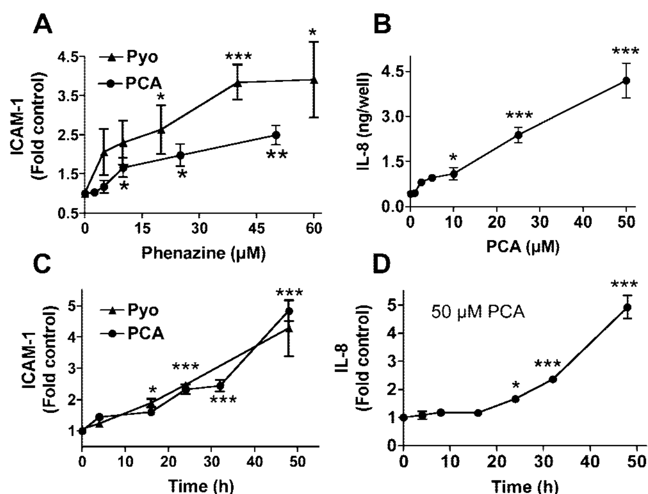

FIGURE 2. Effect of phenazines on ICAM-1 and IL-8. *A* and *B*, A549 cells were treated for 48 h with the indicated concentration of PCA (●) or pyocyanin (▲). *C* and *D*, A549 cells were treated with 50 μM PCA (●) or pyocyanin (▲) for the indicated time. At the end of the incubation period, medium was assayed for IL-8, and cells were assayed for ICAM-1 using ELISA. To combine data from independent experiments, values for ICAM-1 are expressed as fold control, in which control was no phenazine (*A*) or the 0-h time point (*C*), respectively. *D*, To illustrate the lag before PCA-dependent increases in IL-8, data are expressed as fold control relative to basal IL-8 at each comparable time point. All data represent the mean ± SEM (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Similar results were observed in three independent experiments.

Effect of phenazines on the response to host cytokines

Proinflammatory factors are often regulated by multiple stimuli, and additivity or synergy is frequently observed. In this respect, pyocyanin synergizes with host inflammatory cytokines (TNF-α, IL-1β) in increasing IL-8 (23). To determine whether phenazines were additive or synergistic with host cytokines in increasing ICAM-1 and IL-8, A549 cells were incubated with cytokines alone or cytokines together with 50 μM PCA or pyocyanin. As previously reported (23), pyocyanin synergized with TNF-α and IL-1β in increasing IL-8 (data not shown). Similarly, pyocyanin synergized with each cytokine tested in increasing ICAM-1 (Fig. 3*A*): fold increases for cytokines plus pyocyanin were greater than the sum of the individual increases (data not shown). PCA also significantly enhanced TNF- and IFN-, but not IL-1-dependent increases in ICAM-1 (Fig. 3*B*), as well as TNF- and IL-1-dependent increases in IL-8 (Fig. 3*C*). PCA-dependent increases over maximal cytokine values, however, were consistently less robust than those of pyocyanin and may reflect additivity rather than a synergy. Finally, both phenazines increased IL-8 release by polarized monolayers of human airway epithelial cells treated with a cytokine mixture (TNF-α, IL-1β, IFN-γ) (data not shown).

Effect of cycloheximide on phenazine-dependent increases in ICAM-1

To determine whether increases in surface expression of ICAM-1 in vitro were due to protein biosynthesis rather than to mobilization of intracellular stores, A549 cells were pretreated for 1 h with cycloheximide; cycloheximide was also present for the first 6 h of a 24-h treatment period. Cycloheximide treatment abolished the increase in ICAM-1 expression in response to both PCA and pyocyanin (data not shown), suggesting that the increases required protein biosynthesis.

Effect of chemical antioxidants on phenazine-dependent increases in IL-8 and ICAM-1

Pyocyanin-dependent increases in IL-8 are blocked by the thiol antioxidant NAC (23). To examine further whether PCA and/or pyocyanin increase ICAM-1 and/or IL-8 via oxidant-dependent mechanisms, we tested the effect of NAC, DMTU, DMSO, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The concentrations of antioxidants used throughout these studies were not cytotoxic to the cells (see *Materials and Methods* for cytotoxicity assay).

NAC inhibited pyocyanin-dependent increases in ICAM-1 (Fig. 4*A*) and IL-8 (Fig. 4*C*) in a concentration-dependent manner; the dotted line in each case represents control levels. NAC also inhibited PCA-dependent increases in ICAM-1 (Fig. 4*B*) and IL-8 (Fig. 4*D*), but the dose curves for NAC suggested that the response to pyocyanin was considerably more sensitive. Consistent with this conclusion, cumulative data expressed as percentage of inhibition, as well as experiments in which both phenazines were tested in

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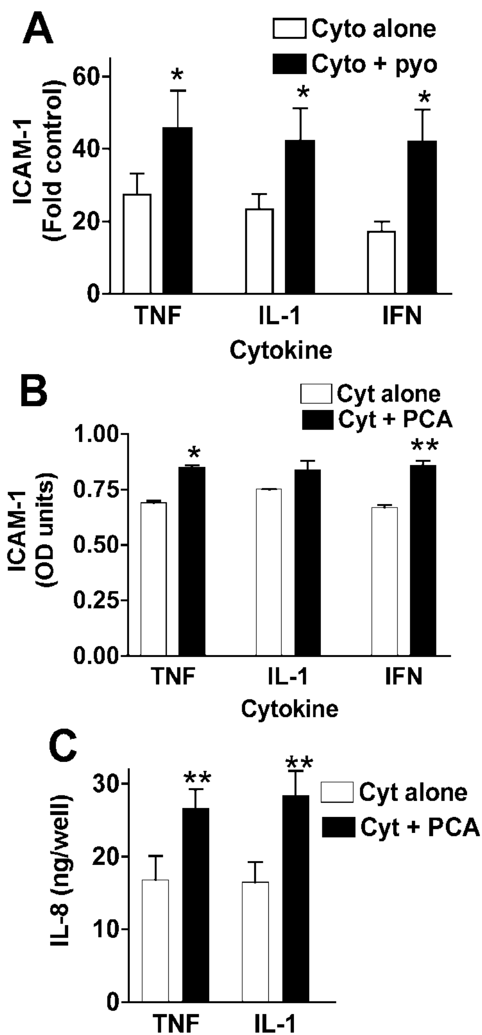

FIGURE 3. Effect of phenazines on cytokine-dependent increases in IL-8 and ICAM-1. A549 cells were treated for 48 h with the indicated cytokine (10 ng/ml TNF-α, 10 ng/ml IL-1β, or 500 U/ml IFN-γ) without (□) or with (■) 50 μM of the indicated phenazine. Medium was assayed for IL-8, and cells were assayed for ICAM-1 using ELISA. *A*, To combine data from two independent experiments, values for ICAM-1 are expressed as fold untreated control. Data represent the mean ± SEM (n = 3–6); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B* and *C*, Similar results were observed in three independent experiments.

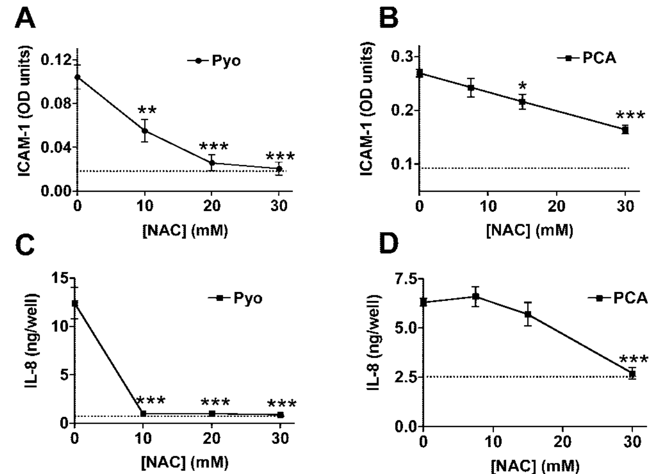

FIGURE 4. Effect of the thiol antioxidant NAC. A549 were pretreated for 1 h with the indicated concentration of NAC. Cultures were then treated without (dotted line, control) or with 50 μM pyocyanin (*A* and *C*) or PCA (*B* and *D*) for 48 h in the continued presence of NAC. At the end of the incubation period, medium was assayed for IL-8, and cells were assayed for ICAM-1 using ELISA. Data represent the mean ± SEM (n = 3); *, $p <$ 0.05; **, $p < 0.01$; ***, $p < 0.001$. Similar results were observed in three independent experiments.

parallel, showed a reproducible statistically significant difference in the effect of NAC on PCA and pyocyanin (data not shown). The response to pyocyanin was also significantly more sensitive to NAC in studies with 16-HBE14o- and NHBE cells (data not shown).

As with NAC, DMTU inhibited pyocyanin-dependent increases in IL-8 and ICAM-1 in a concentration-dependent manner (Fig. 5, *A* and *C*): higher concentrations of DMTU were cytotoxic to the cells. Somewhat surprisingly, DMTU had little or no effect on the response to PCA in A549 cells (Fig. 5, *B* and *D*) or in 16-HBEo- or NHBE cells (data not shown). Two other hydroxyl radical scavengers, DMSO and DMPO, showed a similar pattern of inhibition, i.e., inhibition of the response to pyocyanin, but not to PCA (data not shown).

Effects of NOS inhibitors

A549 cells constitutively produce low levels of NO measured as nitrite and nitrate in the medium. Moreover, human airway epithelial cells produce NO in vivo. Because NO has both pro- and anti-inflammatory effects (28, 29), we wondered whether NO might play a role in the response to phenazines. To test this hypothesis, we treated cells with and without the NOS inhibitor L-arginine methyl ester (L-AME) (Fig. 6). We found that L-AME inhibited the response to both PCA and pyocyanin in a concentration-dependent manner. This suggests that NO contributes to phenazine-dependent increases in IL-8 and ICAM-1.

Effects of antioxidant enzymes

Using fluorescence microscopy techniques, pyocyanin-dependent production of oxidants in A549 cells was shown to occur at or near the plasma membrane, as well as at or near the mitochondria (30). Based on these studies, we next tested whether increasing extracellular antioxidant levels altered phenazine-dependent increases in IL-8 and ICAM-1.

Pyocyanin inactivates human and bovine catalase by oxidizing the NADPH present at the active site (31). In light of these findings, studies of exogenously added antioxidant enzymes were done using catalase from *A. niger*. *A. niger* catalase does not have NADPH at the active site, and its activity is not inhibited by pyocyanin (31). Under these conditions, SOD alone had no effect or enhanced ICAM-1 and IL-8 expression (data not shown). Conversely, we found that extracellular catalase alone or in combination with SOD inhibited phenazine-dependent increases in ICAM-1 (Fig. 7*A*) and IL-8 (Fig. 7*B*) by A549 cells, as well as increased expression of IL-8 in 16-HBE14o- cells and primary cells (data not shown). As expected, under these same conditions, catalase completely inhibited the response to 1 mM H_2O_2 (data not shown).

In studies using adenoviral vectors to overexpress human antioxidant enzymes mitochondrial SOD, cytosolic SOD, and/or catalase (peroxisomal), we observed a small stimulatory effect by SOD and a small inhibitory effect by catalase on pyocyanin-dependent IL-8 release (data not shown). Conversely, phenazine- and cytokine-dependent ICAM-1 expression was not affected by increasing intracellular antioxidant levels.

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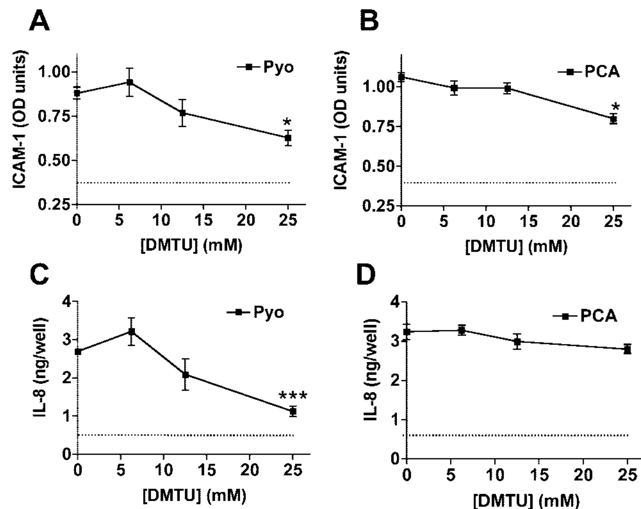

FIGURE 5. Effect of the hydroxyl radical scavenger DMTU. A549 cells were pretreated for 1 h with the indicated concentration of DMTU. Cultures were then treated without (dotted line, control) or with 50 μM pyocyanin (*A* and *C*) or PCA (*B* and *D*) for 48 h in the continued presence of DMTU. Medium was assayed for IL-8, and cells were assayed for ICAM-1 using ELISA. Data represent the mean ± SEM ($n = 3$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Similar results were observed in three independent experiments.

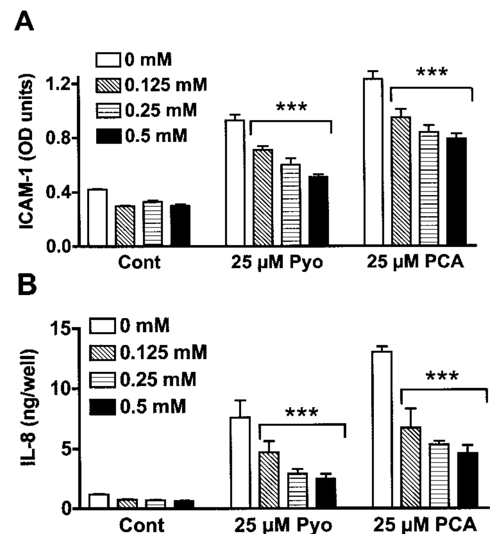

FIGURE 6. Effect of the NOS inhibitor L-AME. A549 were pretreated with the indicated concentration of L-AME for 1 h, and cultures were then incubated for 48 h without (Cont) and with 25 μM PCA or pyocyanin in the continued presence of L-AME. At the end of the incubation period, cells were assayed for ICAM-1 (*A*), and medium was assayed for IL-8 (*B*) using ELISA. Data represent the mean ± SEM ($n = 3$); ***, $p < 0.001$ relative to phenazine alone. Similar results were observed in two independent experiments.

Discussion

The Gram-negative bacterium *P. aeruginosa* is associated with both chronic and acute opportunistic infections in susceptible patient populations with high rates of morbidity and mortality (3, 4). A number of virulence factors expressed by the bacterium have been identified that cause direct damage to host tissues or that contribute to host tissue damage by impacting the host immune response (5). Among *P. aeruginosa* virulence factors are the phenazines pyocyanin, PCA, and 1-hydroxyphenazine. Recent studies by Lau et al. (19) using wild-type and phenazine-deficient strains show that pyocyanin-deficient mutants (*phz*M, *phz*S) are less virulent than wild-type controls in a mouse pneumonia model. This is the first direct evidence of a role for phenazines in the pathophysiologic effects of the bacterium in vivo. Of considerable interest was the finding that pyocyanin-producing strains persisted to a greater extent relative to pyocyanin-deficient strains in a coinfection model, suggesting that pyocyanin conferred a growth/survival advantage for the organism in the lung.

Pyocyanin at physiologically relevant concentrations (13) stimulates a neutrophilic inflammatory response in vivo and increases IL-8 expression by airway cells in vitro (23, 27). Our studies are the first to demonstrate that PCA also stimulates a neutrophilic inflammatory response in a murine model of lung infection, and that both PCA and pyocyanin increase in vivo expression of ICAM-1 and of the murine neutrophil chemokines KC and MIP-2.

ICAM-1 is a major proinflammatory protein up-regulated in both normal and CF airways during bacterial infection (32, 33). We found that both PCA and pyocyanin increased ICAM-1 expression by human airway epithelial cells in vitro, suggesting that increased ICAM-1 expression in vivo may be due, at least in part, to a direct effect on the epithelial cells. Moreover, phenazines enhanced the expression of ICAM-1 and IL-8 in response to maximal concentrations of host cytokines, suggesting differences between phenazines and cytokines in their mechanisms of action. Preliminary studies suggest that the enhanced effect may be due, in part, to enhancing cytokine-dependent activation of NF-κB.

In the *Pseudomonas*-infected lung, both PCA and pyocyanin, as well as other phenazines, may be simultaneously present. When we studied the effects of PCA and pyocyanin together, we found that the combination elicited similar increases in IL-8 and ICAM-1 relative to the same total concentration of each phenazine alone (data not shown). This suggests that PCA and pyocyanin may increase expression of these two proteins via similar mechanisms. However, we also found that phenazine combinations were more cytotoxic than comparable concentrations of individual phenazines, suggesting that each phenazine has additional independent effects that when combined have a greater impact on cell viability.

Fig. 8 demonstrates a model that summarizes our current knowledge. Both PCA and pyocyanin increase intracellular oxidant formation (11, 12). However, whereas pyocyanin can redox cycle using simple reducing sources (NADPH, thiols), PCA cannot (20). Thus, alternative mechanisms for oxidation of PCA must be involved. In addition, we cannot rule out the possibility that the cell converts PCA to a compound that is responsible for oxidant formation. Furthermore, recent studies indicate that oxidant formation by pyocyanin may occur both at the plasma membrane and near the mitochondria (30). Whether PCA generates oxidants at the same sites as pyocyanin or at different sites remains to be determined. These differences in the location and/or mechanisms of redox cycling may explain, in part, the differences in the ability of antioxidants to inhibit PCA- and pyocyanin-mediated effects.

Relatively little is known about the signaling pathways activated by *P. aeruginosa* phenazines that lead to downstream effects, including effects on gene expression. Unlike bacterial products that act through receptors on host cell membranes, phenazines and their radical forms are thought to readily diffuse into and out of the cell, and thus, could directly or indirectly (e.g., by reactive oxygen species production) exert their effects at multiple points in cellular signaling pathways. Additionally, not all of the effects of pyocyanin are blocked by antioxidants (34), and we must therefore consider the possibility that some phenazine-dependent effects on gene expression are oxidant-independent.

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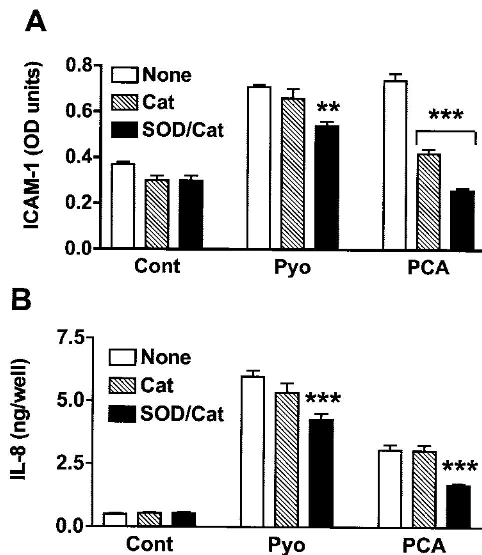

FIGURE 7. Effect of extracellular antioxidant enzymes. A549 cells were treated for 48 h without and with 50 μM PCA or pyocyanin in the absence (None) or presence of the indicated antioxidant enzyme(s): 100 U/ml bovine erythrocyte SOD, 0.4 μM *A. niger* catalase (Cat), or both (Cat/SOD). At the end of the incubation period, medium was assayed for IL-8 (*B*), and cells were assayed for ICAM-1 (*A*) using ELISA. Data represent the mean ± SEM ($n = 6$); **, $p < 0.01$; ***, $p < 0.001$ relative to no antioxidant enzyme controls. Similar results were seen in three independent experiments.

To assess the potential role of oxidants in phenazine-mediated gene expression, we used chemical antioxidants, inhibitors of NO synthase, and antioxidant enzymes. The thiol antioxidant NAC inhibited the response to both PCA and pyocyanin. NAC also inhibited the response to exogenous H_2O_2, TNF-α, and IL-1β (data not shown). NAC may exert its anti-inflammatory effect by a number of mechanisms, including direct inhibition of NF-κB and other oxidant-sensitive transcription factors (35). Together, our data indicate that NAC exerts anti-inflammatory effects in response to a variety of stimuli and might therefore be promising for treating chronic infection with *P. aeruginosa*.

Of particular interest was the observation that the pyocyanin response was considerably more sensitive to NAC. Moreover, we observed that medium with pyocyanin and NAC developed a brown color over time. If this medium was chloroform extracted, no pyocyanin was recovered. Conversely, pyocyanin in the absence of NAC was recoverable from growth medium under identical conditions. As pyocyanin can directly oxidize NAC, as well as reduced glutathione (36), we speculate that the product formed from pyocyanin and NAC may have reduced redox activity. Preliminary data using electron paramagnetic resonance spectroscopy support this conclusion (data not shown). These findings suggest that NAC may have the added benefit of directly inactivating pyocyanin. Conversely, PCA does not react directly with thiol compounds, and thus, no comparable benefit would be expected.

DMTU, DMSO, and DMPO are hydroxyl radical scavengers. All three compounds inhibited the response to pyocyanin to a significantly greater degree than the response to PCA. This suggests that PCA and pyocyanin may differ in their overall pattern of ROS formation or that pyocyanin-generated oxidants are more accessible to these compounds. With respect to other stimuli, DMTU completely inhibited the response to H_2O_2, but had little or no effect on the response to TNF-α and IL-1β (data not shown). Thus, the hydroxyl radical scavengers may be less effective in reducing inflammation in vivo when compared with NAC.

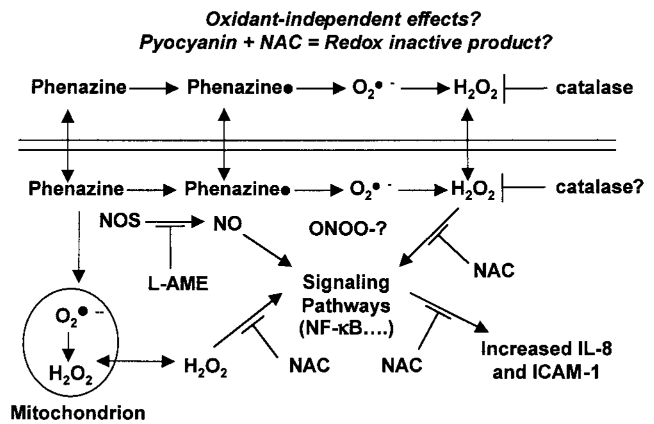

FIGURE 8. Proposed model of phenazines and antioxidants.

Both reactive oxygen species and reactive nitrogen species alter signaling pathways. We found that the NOS inhibitor L-AME inhibited the response to both PCA and pyocyanin. In this respect, phenazine-dependent superoxide formation might scavenge NO and thus prevent its anti-inflammatory effects (37). In addition, we cannot rule out the possibility that the product of superoxide and NO, peroxynitrite, might directly contribute to phenazine-dependent increases in IL-8 and ICAM-1 expression.

We also studied the effect of antioxidant enzymes on the response to phenazines. An important caveat in studies with pyocyanin is its ability to inactivate human catalase (31). For this reason, *A. niger* catalase was used in studies of exogenous enzyme addition. Extracellular SOD alone enhanced the response to both PCA and pyocyanin. This suggests that superoxide is generated outside the cell and that increasing its conversion to H_2O_2 has a proinflammatory effect. Conversely, extracellular catalase with and without SOD inhibited the response to phenazines, supporting a proinflammatory role for extracellular H_2O_2 and suggesting that phenazine-dependent oxidant formation at or near the plasma membrane may contribute to increased expression of IL-8 and ICAM-1. Conversely, overexpressing antioxidant enzymes within the cell using adenoviral constructs had little or no effect on the response to phenazines or to host cytokines. These data suggest that increasing the antioxidant potential of cells using adenoviral vectors may prove of limited therapeutic value.

Numerous questions regarding the mechanism of phenazine action remain. Addressing these questions will be of considerable importance to fully understand the mechanisms by which *P. aeruginosa* causes lung disease. Understanding these mechanisms will in turn be essential to designing targeted therapeutic approaches for treating these infections.

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Disclosures

The authors have no financial conflict of interest.

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