Subcellular localization of *Pseudomonas* pyocyanin cytotoxicity in human lung epithelial cells

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1Research Service and 4Department of Internal Medicine, Iowa City Veterans Affairs Medical Center, Iowa City 52246; and 2Department of Internal Medicine and 3Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa 52242

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O’Malley, Yunxia Q., Maher Y. Abdalla, Michael L. McCormick, Krzysztof J. Reszka, Gerene M. Denning, and Bradley E. Britigan. Subcellular localization of *Pseudomonas* pyocyanin cytotoxicity in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 284: L420–L430, 2003. First published November 1, 2002; 10.1152/ajplung.00316.2002.—The pathogenic mechanism(s) in chronic lung infections in patients with cystic fibrosis (CF) or chronic bronchiectasis (12, 13, 34, 44). Chronic lung injury is currently the primary cause of death in CF and has been linked to coexistent *P. aeruginosa* infection (12, 13). The pathogenic mechanism(s) involved in *P. aeruginosa*-mediated tissue damage in the lung remain uncertain (12, 13, 34, 44).

Most *P. aeruginosa* strains secrete pyocyanin (N-methyl-1-hydroxyphenazine, mw 210) (54). Pyocyanin is among the *P. aeruginosa* cytotoxic secretory factors that are felt to contribute to organism virulence (12, 13, 33, 34, 44, 51, 52). Pyocyanin’s cytotoxicity has been linked to its propensity to undergo cell-mediated redox cycling with resultant formation of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (5, 6, 8, 14, 17, 21–23, 29, 37, 46). Both NADH and NADPH directly reduce pyocyanin (5, 14, 17, 23). Under aerobic conditions, electrons are readily transferred from the reduced form of pyocyanin to O$_2$ thereby generating O$_2^-$ and H$_2$O$_2$.

Pyocyanin can be detected in pulmonary secretions of CF patients and other individuals with chronic bronchiectasis who are infected with *P. aeruginosa* at concentrations of up to 100 μM (56). These concentrations of pyocyanin cause a variety of deleterious effects on pulmonary cells and tissues in vitro and in vivo (1, 10, 27, 28, 39, 47, 55, 56). Protection by pharmacological agents suggests that at least some of these deleterious effects result from pyocyanin-mediated depletion of cellular cAMP and ATP (11, 28) that occurs via oxidant production (27, 47).

Perhaps linked to cellular depletion of ATP, experiments using either respiratory tissue or the A549 type II alveolar cell line suggest that pyocyanin may damage mitochondria (11, 14, 17). In the case of the cell line, data suggested that pyocyanin-mediated inhibition of cellular aconitase might be important (14, 17). Inhibition of aconitase is not surprising given its known susceptibility to inactivation by O$_2^-$ (15, 16, 18, 19, 24, 32). However, to what extent both cytoplasmic and mitochondrial aconitase is affected was not directly examined (14, 29). Furthermore, the assay used to measure aconitase activity relies on the detection of NADPH production from NADP$^+$ by aconitase. Because pyocyanin directly oxidizes NADPH to NADP$^+$, there is the possibility that this assay could overestimate the effect of pyocyanin on cellular aconitase activity. Early work in tumor cells showed that pyocyanin-mediated cell death was linked to increased aconitase activity (57). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Pyocyanin decreases succinate dehydrogenase (SDH) activity (49), another mitochondrial enzyme, suggesting that it too could be involved in pyocyanin-mediated depletion of ATP.

The site(s) of $O_2^-$ and $H_2O_2$ generation within a cell is a key factor in determining its effect(s) on cellular function(s). To date there has been little assessment of the sites within eukaryotic cells where reactive oxygen species (ROS) are generated on exposure to pyocyanin. $O_2^-$ formation, detected using spin trapping techniques following exposure of cells to pyocyanin, appeared to occur predominantly in the extracellular environment (5). However, effects of pyocyanin on cellular mitochondrial function would imply production of ROS in close proximity to those structures.

Therefore, we conducted a series of experiments whose goal was to further define the site of pyocyanin redox cycling within airway epithelial cells with a particular emphasis on the impact of such events on cellular mitochondrial function and energy (ATP) production.

**MATERIALS AND METHODS**

**Cell culture.** The human alveolar type II cell line A549 [CL-185; American Type Culture Collection (ATCC), Rockville, MD] was cultured in Dulbecco's modified Eagle's medium (Cellgro) containing 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 500 U/ml each of penicillin and streptomycin. Passages from 73 to 100 were used.

**Serum medium.** Serum-free medium (modified Eagle's medium; [CL-185; American Type Culture Collection (ATCC), Rockville, MD]) and Millipore membrane. After this incubation, the cells were collected and sonicated with Tris buffer (50 mM, pH 7.4) containing protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany), and the cell lysate was separated from cellular debris by centrifugation (14,000 g, 1 min).

For differentiation of cytosolic from mitochondrial aconitase activity, the cells were suspended in Tris (50 mM, pH 7.4) buffer containing 70 mM sucrose, 210 mM mannitol, and protease inhibitor cocktail tablet (Boehringer) and placed on ice. The cell membrane was disrupted by nitrogen cavitation (200 psi for 5 min). The supernatant (cytosolic aconitase fraction) and pellet (mitochondrial aconitase fraction) were separately centrifuged at 10,000 g, 10 min. The separation of cytosol and mitochondria was confirmed by immunoblotting the fractions for the presence of MnSOD (mitochondrial marker) and CuZnSOD (cytosolic marker).

**Confocal microscopy.** A549 cells were cultured to confluence in six-well plates. Cells were treated with 20 μM pyocyanin for 2 h in Hank's balanced salt solution (HBSS). A fresh standard buffer was made of 0.1 M Tris-maleate buffer, pH 7.4, with 7% sucrose (TMB/S). Cells were incubated with TMB/S with 1 mM cerium chloride (CeCl₃; Sigma), 10 mM aminothiazole, and 0.71 mM NADH for 1 h at 37°C. All solutions were sterilized by filtration through a 0.45-μm Millipore membrane. After this incubation, the cells were fixed in 2% glutaraldehyde in 0.1 mM cacodylate buffer, pH 7.4, at 4°C for 60 min. After being washed with TMB/S, cells were postfixed in 1% OsO₄ at 4°C for 60 min, dehydrated in graded ethanol, and embedded in Spurr’s resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined by electron microscopy under a Hitachi H-600 electron microscope. Controls included cells treated with pyocyanin alone, CeCl₃ alone, and buffer alone.

**Aconitase and succinate dehydrogenase activity.** For determinations of total aconitase and SDH activity, the cells were collected and sonicated with Tris buffer (50 mM, pH 7.4) containing protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany), and the cell lysate was separated from cellular debris by centrifugation (14,000 g, 1 min).

The protein content of each sample was determined using the BCA protein assay (Pierce, Rockford, IL). For aconitase determinations, 50 μg of protein from the supernatant were mixed with reaction buffer [50 mM Tris (pH 7.4), 1 U/ml isocitrate dehydrogenase, 0.6 mM MnCl₂, 20 μM fluorocitrate, 400 μM cis-aconitate, 1 μM phenazine methosulfate (PMS), 200 μM cytochrome c, and 200 μM NADP⁺], and the mixture was incubated at 25°C for 30 min. The optical density (OD) was measured at 550 nm. In some assays for mitochondrial aconitase activity, 1 mM NaN₃ was added to the reaction mixture to prevent reoxidation of cytochrome c by mitochondrial cytochrome oxidase.

For SDH determinations, 50 μg of protein from the supernatant were mixed with a different reaction buffer [50 mM Tris (pH 7.4), 20 mM sodium succinate, 1 μM KCN, 1 μM PMS, and 5 mM cytochrome c], and the OD was measured at 550 nm after 5 min at 25°C.

**Immunoblotting.** Cells were rinsed twice with PBS and lysed by addition of Tris buffer (50 mM, pH 7.4) containing 1% Nonidet P-40 (Amresco, Solon, OH). Cell lysates were collected into Eppendorf tubes and sonicated, and cellular debris was removed by centrifugation (14,000 g, 1 min). Samples (20–30 μg protein) were mixed 1:1 with sample buffer (1.25 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.05% bromphenol blue), and proteins were separated by 10% SDS-PAGE. The protein was transferred to a nitrocellulose membrane overnight at 30 V. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween (TBST) for 1 h and incubated with the primary antibody (1:1,000 dilution) for 1–2 h. The blot was washed with TBST and incubated with a 1:10,000 dilution of the secondary antibody (horseradish peroxidase-conjugated anti-IgG; Amersham Pharmacia Biotech, Piscataway, NJ). The immunoreactive protein was detected...
with an enhanced chemiluminescence detection kit (Amer-
sham Pharmacia Biotech).

**ATP determination.** The procedure was adapted from that
of Takahashi et al. (50) with some modification. We obtained
the cell extract by adding 100 μl of ice-cold perchloric acid
(6%) to cells. The perchloric acid solution was collected in a
test tube and neutralized to pH 7 with 22.5 μl of KOH (4 M)
and 10 μl of Tris buffer (2 M). ATP was measured by the
luciferin-luciferase method with a luminometer (Analytical
Luminescence Laboratories, Cockeysville, MD) according to
the manufacturer’s instructions. Results were normalized to
cellular protein content. Results were similar when cellular
DNA was used as the denominator.

**DNA quantification.** The cells were lysed with Tris buffer
(50 mM, pH 7) containing 10 mM NaCl and 0.1% SDS. The
cell solution (1 ml) was mixed with 10 μl of 100 μg/ml
4’,6-diamidino-2-phenylindole and incubated at RT for 10
min. The fluorescent intensity was measured at an excitation
wavelength of 360 nm and emission wavelength of 460
nm (30).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bro-
mide reduction. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl
tetrazolium bromide (MTT) was dissolved in MEM without
phenol red. A549 cells were incubated with MTT (0.5 mg/ml)
at 37°C for 2 h. They were then lysed with 100% propanol. We
then determined the presence of blue formazan resulting
from cell-mediated reduction of MTT by measuring absor-
bance at 550 nm.

**SOD and catalase overexpression.** Recombinant adenoviral
vectors expressing MnSOD (Ad CMV MnSOD), CuZnSOD
(Ad CMV CuZnSOD), catalase (Ad CMV catalase), or β-ga-
lactosidase (Ad CMV LacZ) were constructed by and pur-
chased from the Vector Core Facility of The University of
Iowa. Each adenoviral stock (4–6 × 109 DNA particles/ml)
was stored in 3% sucrose at −80°C. Multiplicities of infection
(MOI) ranging from 10 to 100 were routinely employed. Cells
were exposed to adenovirus in media containing 5% FBS at
37°C for 24 h. We confirmed successful transfection by mea-
suring expression of each antioxidant enzyme at both the
protein and activity level by immunoblot and by a native gel
activity assay staining, respectively (3, 4, 35).

**Pyocyanin metabolism.** Increasing concentrations of pyo-
cyanin were added to A549 monolayers or cell-free media,
and samples were incubated for 4, 8, 12, and 24 h. Cells and
medium were harvested from the wells, pyocyanin was ex-
tracted with chloroform, and the amount of pyocyanin recov-
ered was then quantified by HPLC as described in Pyocyanin
purification.

**Mitochondrial potential.** Mitochondrial membrane poten-
tial was measured fluorometrically by previously described
methods (7, 53). Briefly, the cells were grown in 96-well
plates and incubated with or without pyocyanin for 24 h. The
medium was removed, and the wells were rinsed twice with
HBSS. The fluorescent probe JC-1 dissolved in HBSS
(2 μg/ml; Molecular Probes, Eugene, OR) was incubated with
the cells for 4 h at 37°C. Fluorescence intensity was mea-
sured at excitation and emission wavelengths of 544/590 nm
(red) and 485/530 nm (green). Mitochondrial membrane poten-
tial is proportional to the red/green fluorescence intensity ratio.

**RESULTS**

**Subcellular localization of pyocyanin redox cycling.** Pre-
vious work indicates that pyocyanin redox cycles on expo-
sure to either eukaryotic or prokaryotic cells, re-
resulting in the production of O₂•− and H₂O₂ (5, 6, 8, 14,
21–23, 29, 37, 46). However, the subcellular site(s) at
which this redox cycling occurs in eukaryotic cells has
not been extensively investigated. To investigate this
in human airway epithelial cells, A549 cell monolayers
were loaded with the oxidant-sensitive probe DCFH₂,
exposed to pyocyanin, and then examined by confocal
microscopy. When DCFH₂ is oxidized to DCF there is
an increase in fluorescence (2, 26, 31, 45). Such oxida-
tion can result from the interaction of DCFH₂ with
products of the reaction of H₂O₂ and intracellular per-
oxidases, making it a popular detection system for
changes in intracellular ROS levels (26).

As shown in Fig. 1, when DCFH₂-loaded A549 cells
were exposed to pyocyanin, an increase in intracellular
fluorescence was observed that increased with the con-
centration of pyocyanin employed. The fluorescence
appeared to be concentrated in the perinuclear region
of the cell (Fig. 1). This pattern of fluorescence is
consistent with mitochondrial localization. To test this
possibility, the cells were loaded with both an oxidant-
insensitive fluorescent probe that localizes to mito-
hondria (Mitotracker red) and DCFH₂. The cells were then
exposed to pyocyanin under these conditions. DCF fluo-
rescence colocalized with Mitotracker red (Fig. 1), suggest-
ing that DCFH₂ oxidation resulting from cellular exposure to
and redox cycling of pyo-
cyanin occurs in close proximity to cellular mitochondria.

Although the pattern of DCF fluorescence suggested
that these species are likely formed in or near the
mitochondria, subsequent studies indicated that pyo-
cyanin-induced H₂O₂ formation can also be detected at
other sites as well. Exposure of the cells to the mem-
brane-impermeable compound CeCl₃ in the presence of
H₂O₂ results in the formation of an electron-dense precipi-
tate, Ce(OH)₃OOH, whose presence can be de-
termined at/near the mitochon-
dria (Mitotracker red) and DCFH₂. When A549 cells were
exposed to pyocyanin, an increase in intracellular CeCl₃,
an electron-dense precipi-
tate was observed at the plasma membrane (Fig. 2). We
also observed ultrastructural changes in A549 cell mi-
thochondria after 2 h of pyocyanin exposure, but only at
the 100 μM concentration (Fig. 3).

Thus the combination of confocal and electron mi-
croscopy studies indicates that intracellular redox cy-
cling of pyocyanin occurs in close proximity to the
mitochondria. H₂O₂ production also occurs and can be
detected outside the cell. Whether the H₂O₂ detected
extracellularly arose as a consequence of pyocyanin
redox cycling that occurred first at/near the mitochon-
dria and then diffused cannot be determined from
these studies.

**Pyocyanin depletes cellular ATP and alters mito-
chondrial electron transport.** Because mitochondrial
activity is critical to optimal production of cellular
ATP, we sought to confirm previous reports (11, 28)
that pyocyanin exposure depletes cells of ATP. Consis-
tent with these previous reports, we found a time-
and concentration-dependent depletion of the ATP content
of cells exposed to pyocyanin (Fig. 4).
Cellular reduction of MTT to formazan is thought to reflect mitochondrial activity (48). Exposure of A549 cells to pyocyanin resulted in a time- and dose-dependent decrease in their capacity to reduce MTT (Fig. 5). These results were not due to the loss of the cellular monolayer viability as measured by DNA content of the wells (data not shown).

Pyocyanin inhibits mitochondrial and cytosolic aconitase activity. Given that mitochondrial electron transport is critical to cellular ATP production and that pyocyanin redox cycling appeared to occur at/near mitochondria, and that pyocyanin decreased cellular MTT reduction, we sought to explore further whether mitochondria were targets of pyocyanin’s effects and, if so, what mitochondrial components were affected.

Previous work by Gardner and colleagues (14, 17) demonstrates that aconitase activity is inhibited in A549 cells by pyocyanin. This is not surprising given that the Fe-S center of aconitase is extremely sensitive to the effects of $O_2^-$ (18). However, the aconitase activity assay employed in this previous work measures the aconitase-dependent production of NADPH from NADP+ by cell lysates (18, 19). Given that pyocyanin directly oxidizes NADPH and/or NADH (5, 22, 23), we were concerned that, if cellular lysates from pyocyanin-exposed cells contained residual pyocyanin, that pyocyanin might directly consume NADPH being produced by the enzyme during the aconitase activity assay. This would lead to an underestimation of aconitase activity and a corresponding increase in the apparent inhibitory effect of pyocyanin on aconitase activity. Consistent with the work by Gardner and colleagues (14, 17), when we used the standard aconitase assay following exposure of the cells to pyocyanin,
concentrations as low as 2 μM resulted in a significant decrease in apparent enzyme activity (data not shown). However, in support of the stated concern outlined above, addition of cellular lysate from pyocyanin-treated cells to a solution of purified aconitase decreased apparent activity of the enzyme over the course of the assay. The lysate also possessed the ability to consume NADPH when added to a known concentration of NADPH, as monitored spectrophotometrically over time (not shown).

Given the above findings, we chose an alternative approach for determining aconitase activity. This approach, based on a native gel activity assay system (38), was modified to be used in a spectrophotometric assay. This assay also relies on the ability of aconitase to generate NADPH, but instead of detecting accumulated NADPH, it uses the NADPH as a reducing source through which PMS, a compound with many structural similarities to pyocyanin, converts an oxidized detector molecule to its reduced form (38). The original assay utilized reduction of nitro blue tetrazolium (NBT). We chose to use ferricytochrome c in place of NBT because reduced cytochrome c remains water soluble, whereas reduced NBT forms insoluble formazan. In addition, we previously showed that pyocyanin also efficiently reduces ferricytochrome c in an NADPH-dependent manner. We chose to use ferricytochrome c in place of NBT because reduced cytochrome c remains water soluble, whereas reduced NBT forms insoluble formazan. In addition, we previously showed that pyocyanin also efficiently reduces ferricytochrome c in an NADPH-dependent manner.

Fig. 2. Pyocyanin exposure leads to the presence of extracellular H₂O₂. A549 monolayers were incubated with buffer (A) or 50 μM pyocyanin (B) for 30 min in the presence of CeCl₃, following which the cells were fixed and examined by transmission electron microscopy for evidence of Ce(OH)_3OOH electron-dense precipitate, indicating the presence of H₂O₂. Pyocyanin increased the amount of extracellular H₂O₂ detected (A vs. B). Results are representative of 3 separate experiments. A and B are at ×60,000 and ×100,000, respectively.

Fig. 3. Effect of pyocyanin on mitochondrial ultrastructure. A549 monolayers were incubated with buffer (A), 50 μM pyocyanin (B), or 100 μM pyocyanin (C) for 2 h, following which the cells were fixed and their mitochondria were examined by transmission electron microscopy (×100,000). At the highest concentration of pyocyanin, a decrease in mitochondrial matrix structure was observed. Results shown are representative of 3 separate experiments.
manner (36), both through the formation of a O₂⁻/H₂O₂ intermediate and via the direct reduction of cytochrome c by reduced pyocyanin (36). Because the reaction of NADPH with either pyocyanin or PMS in the presence of cytochrome c yielded the same reduced cytochrome c product, this approach allows for the measurement of aconitase activity that is unaffected by any pyocyanin that remains in the cell lysates. This approach yielded a linear assay for aconitase activity (Fig. 6A).

Using this alternative aconitase assay, we found that pyocyanin decreased cellular aconitase activity. However, the magnitude of the decrease was less than observed with the previous assay (14, 17). Inhibition of total cellular aconitase plateaued at about 50% (Fig. 6B).

Fig. 4. Pyocyanin exposure decreases ATP levels. A549 monolayers were incubated for 24 h with increasing concentrations of pyocyanin. Cellular ATP content was determined and revealed a concentration-dependent decrease in ATP content relative to control. Values represent the means ± SE ATP concentrations (ng ATP/μg protein, n = 6). Statistically different from no treatment control: *P < 0.05, ***P < 0.01.

Fig. 5. Pyocyanin exposure decreases the ability of A549 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). A549 monolayers were incubated for 1–4 days with increasing concentrations of pyocyanin. Cellular MTT reduction was determined and revealed a concentration-dependent decrease in the ability of pyocyanin-treated cells to reduce MTT relative to control that was maximal at 48 h. Values are expressed as means ± SE % MTT reduction of pyocyanin-treated cells (5, 30, 50, and 80 μM) normalized to untreated control cells (0, open bars) on days 1, 2, 3, and 4 of pyocyanin exposure (n = 6). *P < 0.05, ***P < 0.01, ****P < 0.001 relative to untreated control.

Fig. 6. Pyocyanin exposure decreases A549 cell cytosolic and mitochondrial aconitase activity. A: the linear nature of the assay employed for aconitase activity in which activity is manifested as the reduction of cytochrome (A550, see Aconitase and succinate dehydrogenase activity in MATERIALS AND METHODS). A549 monolayers were incubated for 24 h with increasing concentrations of pyocyanin. Cellular aconitase activity was determined for whole cell lysates (B) or for cytosolic- and mitochondrial-containing fractions (C) and revealed a concentration-dependent decrease in aconitase for total, cytosolic, and mitochondrial aconitase activity. Values are expressed as % of control and represent means ± SE from 12 separate experiments. ***P < 0.001 relative to untreated control.
Cells contain two forms of aconitase, a mitochondrial form and a cytosolic form. The latter is identical to iron response protein-1 (42). Subcellular fractionation of A549 cells revealed that 70% of the total cellular aconitase activity was associated with the cytosolic and 30% with the mitochondrial fractions, values within those previously reported (20). Immunoblot analysis of Mn-SOD and CuZnSOD in the fractions confirmed that our fractionation system efficiently separated the mitochondrion and cytosolic fractions, respectively (data not shown).

We next sought to determine whether pyocyanin-mediated inhibition of total cellular aconitase activity reflected a differential effect on one form of the enzyme. As shown in Fig. 6C, pyocyanin exposure resulted in similar levels of inhibition of cytosolic and mitochondrial aconitase activities. In contrast to mitochondrial aconitase activity, aconitase protein levels in the mitochondria were unaffected by pyocyanin treatment as assessed by immunoblot (data not shown). The absence of an antibody to cytosolic aconitase prevented us from assessing the status of cytosolic aconitase protein levels.

In addition to its inhibition of mitochondrial aconitase, pyocyanin exposure also led to a small, but statistically significant, decrease in the activity of another Fe-S-centered mitochondrial Krebs cycle enzyme, SDH (Fig. 7). SDH activity was measured by a similar modification of the standard SDH activity assay (which also relies on NADPH production) that we used for aconitase measurements (Fig. 7). The inhibition of SDH is consistent with the fact that MTT reduction, which was decreased by pyocyanin, is felt to reflect mitochondrial SDH activity (48).Earlier work reported that SDH activity of some tumor cells was decreased by pyocyanin (49).

Pyocyanin depolarizes mitochondrial membranes. To further assess the effect of pyocyanin on mitochondria, we measured mitochondrial membrane potential. Alteration in mitochondrial membrane potential has been shown to be a sensitive indicator of oxidant damage (53). Using a fluorescent probe-based assay, we observed that exposure of cells to pyocyanin resulted in a decrease in mitochondrial membrane potential (Fig. 8).

Pyocyanin is metabolized by A549 cells. A somewhat surprising observation made during the course of the above experiments was that when the cells were exposed to lower concentrations of pyocyanin, aconitase activity was only transiently decreased, rebounding to normal levels even though the pyocyanin was not washed off the cells following the initial addition (Fig. 9A). This was not due to increases in cellular SOD content (data not shown). These data suggest the possibility that pyocyanin might be undergoing metabolism to a less bioactive compound. Accordingly, pyocyanin was added to A549 monolayers and tissue culture wells lacking cells. At increasing times, the well contents were removed and analyzed for the presence of pyocyanin. In the presence of A549 cells, the pyocyanin concentration decreased, with a half-life of about 12 h (Fig. 9B). This appeared to be a saturable process, as at concentrations >20 μM pyocyanin, the effect was less pronounced (Fig. 9C).

Pyocyanin-induced mitochondrial superoxide activity is linked to aconitase inhibition. Although previous work suggests that pyocyanin-induced O2− production leads to aconitase inhibition (14, 17), the evidence for this is indirect, and the intracellular site responsible for O2− production is not known. To address this question, we assessed the effect of transient transfection of A549 cells with CuZnSOD, MnSOD, or catalase on pyocyanin-dependent inactivation of aconitase activity. CuZnSOD remains cytosolic, whereas MnSOD is expressed in the mitochondria. Transfection of A549 cells with adenovirus expressing CuZnSOD or MnSOD resulted in a marked increase in both the cellular expression of these proteins as well as their enzymatic activity (Fig. 10A for SODs, not shown for catalase) compared with a control vector.

Overexpression of MnSOD (MOI ≥ 30), but not CuZnSOD (MOI 30 or 100) or catalase (MOI 10, 30, or 100), resulted in a decrease in the susceptibility of total cellular aconitase to inactivation by pyocyanin (Fig. 10B for SODs, not shown for catalase). Protection with MnSOD transfection was observed at MOIs of 30 or greater.

Having shown that MnSOD transfection could protect cellular aconitase from inactivation by pyocyanin, we assessed to what extent this process might be responsible for the depletion of ATP by pyocyanin over time. In contrast to its protection of aconitase, MnSOD overexpression did not prevent the pyocyanin-mediated depletion of cellular ATP levels (Fig. 11). Neither CuZnSOD or catalase overexpression prevented the drop in ATP with pyocyanin either (Fig. 11). However, data interpretation of these experiments was confounded somewhat by the apparent decrease of ATP levels in cells transfected with active forms of any of the three antioxidant enzymes (MnSOD, CuZnSOD, or catalase; Fig. 11).

![Fig. 7. Pyocyanin exposure decreases A549 cell succinate dehydrogenase (SDH) activity. A549 monolayers were incubated for 24 h with increasing concentrations of pyocyanin. Cellular SDH activity was determined for whole cell lysates. Values are expressed as the means ± SE percent of control SDH activity from 6 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 relative to untreated control.](Image)
DISCUSSION

Pyocyanin is one of several virulence factors secreted by *P. aeruginosa* (13, 34, 44, 51). Although the ability of pyocyanin to redox cycle and generate ROS has been linked to its cytotoxicity for both eukaryotic and prokaryotic cells, the subcellular targets impacted by pyocyanin remain ill defined. Previous studies suggested mitochondria as one of the targets through which pyocyanin disrupts lung epithelial cell functions and contributes to pathogenesis of lung injury resulting from both acute and chronic *P. aeruginosa* lung infection (14, 17).

Results from confocal microscopy provide the most direct evidence to date that extracellularly administered pyocyanin reaches mitochondria, where it may enhance ROS generation. It is important to note that oxidation of DCFH$_2$ can potentially occur through mechanisms other than ROS production (26, 40, 45). In fact, we have preliminary evidence that suggests that pyocyanin can directly oxidize DCFH$_2$ independently of ROS formation. Thus we cannot definitively attribute the detection of mitochondrial-associated DCF fluorescence to mitochondrial production of O$_2^\cdot$ and/or H$_2$O$_2$. Nevertheless, our results clearly indicate that pyocyanin reaches and redox cycles in, or closely near, cellular mitochondria.

Previous electron microscopy work in which pyocyanin, at a concentration slightly higher than that employed in our studies, was applied to the mucosal surface of human nasal turbinate cultures for 3 h revealed extensive damage to airway cell mitochondria (11). Marked surface blebbing and other changes were also noted (11). In contrast, at 2 h we saw only modest alterations in mitochondrial matrix structure in A549 cells exposed to the highest concentration of pyocyanin that we employed (100 μM). The difference in results from the two studies likely reflects the differences in the model systems, organ culture vs. cell line, antioxidant defenses of the cells involved, as well as perhaps the slightly lower pyocyanin concentrations that we employed. In our experience, A549 cells tend to be more resistant to oxidative injury than other cell types. Nevertheless, our observation that pyocyanin decreases mitochondrial membrane potential supports an impor-

![Fig. 8. Pyocyanin exposure decreases A549 cell mitochondrial membrane potential. A549 monolayers were incubated for 24 h with increasing concentrations of pyocyanin. Cellular mitochondrial membrane potential was then determined. Values shown are the means ± SE percent of control mitochondrial membrane potentials from 12 independent experiments. ***P < 0.001 relative to untreated control.](http://ajplung.physiology.org/)

![Fig. 9. Aconitase activity recovers over 24 h of pyocyanin exposure and correlates with metabolism of pyocyanin by the cells (A). Total cellular aconitase activity of control and pyocyanin-treated (10 and 20 μM) A549 cells was determined at 4, 8, and 24 h of pyocyanin exposure. Aconitase activity returned to near control (left bars) despite the continued presence of 10 μM pyocyanin (middle bars) or 20 μM pyocyanin (right bars). B: pyocyanin (10 μM) was added to tissue culture wells that did (●) or did not (○) contain A549 cells. At indicated time points the pyocyanin concentration in each well was determined. The presence of A549 cells resulted in a loss of detectable pyocyanin over time. C: the experiment reflected was performed as in B except that different concentrations of pyocyanin were added and the magnitude of detectable pyocyanin determined at 24 h in the presence (filled bars) and absence (open bars) of cells. The A549 cell-mediated decrease in pyocyanin was lost at high pyocyanin concentrations. Results shown are representative of 6 separate experiments for A and 4 separate experiments for B and C. *P < 0.05, **P < 0.01, ***P < 0.001 relative to untreated control.](http://ajplung.physiology.org/)
The study investigated the effects of the compound on mitochondria. *P. aeruginosa* exotoxin A has also been shown to alter the mitochondrial morphology of the 16HBE human bronchial epithelial cell line and induce mitochondrial membrane depolarization via a mechanism that involves O$_2^\cdot$ formation (43).

Whether pyocyanin diffuses passively across the cell membrane to reach mitochondria or is actively and selectively directed to this subcellular site remains to be fully defined. Regardless of its mechanism for cellular entry, our studies confirm previous observations that exposure to pyocyanin results in a time- and concentration-dependent depletion of cellular ATP and decreased activity of cellular aconitase, an enzyme known to be highly sensitive to inactivation by O$_2^\cdot$.

However, we find that A549 cell aconitase activity may not be as susceptible to pyocyanin as previously reported (14, 17). This likely reflects our use of an assay that is not susceptible to the confounding effects of residual pyocyanin-mediated consumption of NADPH. Our results confirm previous work indicating that a majority of aconitase activity detected in A549 is cytosolic (20). Surprisingly, both forms of aconitase seemed to be equally susceptible to inactivation by pyocyanin exposure, despite the fact that the confocal microscopy data suggest that a greater degree of pyocyanin redox cycling, and presumably ROS formation, occurs in close proximity to the mitochondria. SDH, another mitochondrial enzyme, was also inhibited by pyocyanin, although to a lesser degree than aconitase. This could be due in part to the fact that, in contrast to aconitase, the Fe-S center of SDH is felt to be buried in the mitochondrial membrane (25).

We found that overexpression of MnSOD (mitochondrial) but not CuZnSOD (cytosolic) protected cellular aconitase activity of the cell. This would suggest that mitochondrial generation of O$_2^\cdot$ is somehow involved in the inactivation of cytosolic aconitase, since much of the total cellular aconitase measured is contributed by the cytosolic fraction. How production of O$_2^\cdot$ at or near the mitochondria affects the activity of the cytosolic enzyme remains to be determined. Nevertheless, since cytosolic aconitase plays a role in regulating cellular iron levels (42), it is possible that pyocyanin could also alter cellular iron metabolism, a possibility that we are currently investigating.

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**Fig. 10.** Overexpression of MnSOD protects A549 cell aconitase from pyocyanin-mediated inhibition. A549 cells were transfected with adenovirus containing CuZnSOD, MnSOD, or lacZ only [100 multiplicity of infection (MOI)]. After 18 h, the cells were incubated for an additional 24 h with and without 50 μM pyocyanin, following which MnSOD and CuZnSOD activity and protein levels were assessed by activity gel and immunoblot, respectively (A), and total aconitase activity was determined (B). Transfection resulted in a marked enhancement of both CuZnSOD and MnSOD expression at both the activity and protein level that was unaltered by pyocyanin treatment (A). MnSOD, but not CuZnSOD, overexpression resulted in a significant decrease in the ability of pyocyanin to inhibit aconitase activity of the cells. Open bars indicate results with nonpyocyanin-treated cells compared with those exposed to pyocyanin, indicated by the filled bars (B). Results are representative of 8 separate experiments. ***P < 0.001 relative to untreated control.

**Fig. 11.** Overexpression of antioxidant does not prevent pyocyanin-mediated depletion of ATP. A549 cells were transfected with adenovirus containing CuZnSOD, MnSOD, catalase, or lacZ only (MOI as noted). After 18 h, the cells were incubated for an additional 24 h with (open bars) and without (filled bars) 50 μM pyocyanin following which cellular ATP content was determined. Results shown are means ± SE ATP content (ng/μg protein) for 6–16 independent determinations. In each case, the ATP content of the cells significantly decreased following pyocyanin treatment. ***P < 0.001 relative to untreated control. However, expression of each of the antioxidant enzymes themselves led to a statistically significant drop in the ATP content of the cells relative to lacZ control (P < 0.001).
Mitochondrial electron transport via the Krebs cycle plays a critical role in cellular ATP production. Because pyocyanin depletes cellular ATP and inhibits a key component of the Krebs cycle (aconitase), it would be logical to link the two events. However, our transfection studies with MnSOD reveal that, despite our ability to protect both mitochondrial and cytosolic aconitase activity from pyocyanin-mediated inhibition, cellular loss of ATP as a consequence of pyocyanin exposure still occurred. However, these data are somewhat difficult to interpret due to the fact that, for unknown reasons, overexpression of MnSOD, as well as CuZn-SOD and catalase, by themselves produced a decrease in the ATP levels of the cells.

Nevertheless, these results suggest that other cellular components, besides aconitase, are negatively affected by pyocyanin and are responsible for the loss of cellular ATP resulting from cellular exposure to this agent. Whether this involves pyocyanin-mediated effects on cellular levels of NADH or NADPH, inhibition of glycolysis, or other mitochondrial processes requires further investigation.

An additional and previously unappreciated aspect of the interaction of pyocyanin with airway epithelial cells is that these cells have the capacity to catabolize pyocyanin. The products of this metabolism are currently under study. Cellular recovery of aconitase activity correlates with the extent to which pyocyanin is metabolized, suggesting that the product(s) of pyocyanin metabolism is less cytotoxic than the parent compound.

We have shown that, when administered to airway epithelial cells in vitro, pyocyanin can move to subcellular sites in which mitochondria reside, where it is able to participate in redox chemistry. Pyocyanin exposure leads to a decrease in cellular ATP and inhibition of both mitochondrial and cytosolic aconitase activity, the extent and persistence of which are influenced by cellular metabolism of pyocyanin. Although augmenting MnSOD levels protects cellular aconitase, this does not prevent pyocyanin-mediated depletion of cellular ATP, suggesting the events are not linked. The way in which pyocyanin exposure leads to depletion of ATP, the mechanism of cellular metabolism of pyocyanin, and the impact of mitochondrial pyocyanin redox cycling on other cellular events are important areas for future study.

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REFERENCES


