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

Yunxia Q. O’Malley, Maher Y. Abdalla, Michael L. McCormick, Krzysztof J. Reszka, Gerene M. Denning and Bradley E. Britigan


You might find this additional info useful...

This article cites 52 articles, 32 of which can be accessed free at:

http://ajplung.physiology.org/content/284/2/L420.full.html#ref-list-1

This article has been cited by 7 other HighWire hosted articles, the first 5 are:

**GSH monoethyl ester rescues mitochondrial defects in cystic fibrosis models**

Mairead Kelly-Aubert, Stéphanie Trudel, Janine Fritsch, Thao Nguyen-Khoa, Maryvonne Baudouin-Legros, Sandra Moriceau, Ludovic Jeanson, Fatima Djouadi, Corine Matar, Marc Conti, Mario Ollero, Franck Brouillard and Aleksander Edelman


[Abstract] [Full Text] [PDF]

**Impairment of Apoptotic Cell Engulfment by Pyocyanin, a Toxic Metabolite of *Pseudomonas aeruginosa***


[Abstract] [Full Text] [PDF]

**Pathogen–Host Interactions in *Pseudomonas aeruginosa* Pneumonia**

Ruxana T. Sadikot, Timothy S. Blackwell, John W. Christman and Alice S. Prince


[Abstract] [Full Text] [PDF]

**Pseudomonas aeruginosa** pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells

Yunxia Q. O’Malley, Krzysztof J. Reszka, Douglas R. Spitz, Gerene M. Denning and Bradley E. Britigan


[Abstract] [Full Text] [PDF]

Updated information and services including high resolution figures, can be found at:

http://ajplung.physiology.org/content/284/2/L420.full.html

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

http://www.the-aps.org/publications/ajplung

This information is current as of May 7, 2012.
First published November 1, 2002; 10.1152/ajplung.00316.2002.

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

YUNXIA Q. O’MALLEY,1,2 MAHER Y. ABDALLA,3 MICHAEL L. MCCORMICK,1–3 KRZYSZTOF J. RESZKA,1,3 GERENE M. DENNING,1,2,4 AND BRADLEY E. BRITIGAN1–4
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

Submitted 17 September 2002; accepted in final form 30 October 2002

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 *Pseudomonas aeruginosa* secretory product pyocyanin damages lung epithelium, likely due to redox cycling of pyocyanin and resultant superoxide and H2O2 generation. Subcellular site(s) of pyocyanin redox cycling and toxicity have not been well studied. Therefore, pyocyanin’s effects on subcellular parameters in the A549 human type II alveolar epithelial cell line were examined. Confocal and electron microscopy studies suggested mitochondrial redox cycling of pyocyanin and extracellular H2O2 release, respectively. Pyocyanin decreased mitochondrial and cytoplasmic aconitase activity, ATP levels, cellular reduction of NAD(P)H, and mitochondrial membrane potential. These effects were transient at low pyocyanin concentrations and were linked to apparent cell-mediated metabolism of pyocyanin. Overexpression of MnSOD, but not CuZnSOD or catalase, protected cellular aconitase, but not ATP, from pyocyanin-mediated depletion. This suggests that loss of aconitase activity is not responsible for ATP depletion. How pyocyanin leads to ATP depletion, 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.

mitochondria; superoxide; hydrogen peroxide; aconitase

*Pseudomonas aeruginosa* causes acute severe necrotizing pneumonia with high mortality (13, 34) as well as 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 (O2·−) and hydrogen peroxide (H2O2) (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 O2, thereby generating O2·− and H2O2. 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 mitochondrial aconitase might be important (14, 17). Inhibition of aconitase is not surprising given its known susceptibility to inactivation by O2·− (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 pyoca-
subcellular localization of pyocyanin cytotoxicity

AJP-Lung Cell Mol Physiol • VOL 284 • FEBRUARY 2003 • www.ajplung.org

...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 aminotriazole, 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). 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 separated by centrifugation (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).

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 NaCN 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 mM 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 M 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 bromide 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 absorbance 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-galactosidase (Ad CMV LacZ) were constructed by and purchased from the Vector Core Facility of The University of Iowa. Each adenoviral stock (4–6 × 10^9 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 (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 transduction by measuring 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 pyocyanin 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 extracted with chloroform, and the amount of pyocyanin recovered at each time point was then quantified by HPLC as described in Pyocyanin purification.

**Mitochondrial potential.** Mitochondrial membrane potential 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 measured at excitation and emission wavelengths of 544/590 nm (red) and 485/530 nm (green). Mitochondrial membrane potential is proportional to the red/green fluorescence intensity ratio.

**RESULTS**

**Subcellular localization of pyocyanin redox cycling.** Previous work indicates that pyocyanin redox cycles on exposure to either eukaryotic or prokaryotic cells, resulting in the production of O_2− and H_2O_2 (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 DCFH_2, exposed to pyocyanin, and then examined by confocal microscopy. When DCFH_2 is oxidized to DCF there is an increase in fluorescence (2, 26, 31, 45). Such oxidation can result from the interaction of DCFH_2 with products of the reaction of H_2O_2 and intracellular peroxidases, making it a popular detection system for changes in intracellular ROS levels (26).

As shown in Fig. 1, when DCFH_2-loaded A549 cells were exposed to pyocyanin, an increase in intracellular fluorescence was observed that increased with the concentration 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-sensitive fluorescent probe that localizes to mitochondria (Mitotracker red) and DCFH_2. The cells were then exposed to pyocyanin under these conditions. DCF fluorescence colocalized with Mitotracker red (Fig. 1), suggesting that DCFH_2 oxidation resulting from cellular exposure to and redox cycling of pyocyanin 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 pyocyanin-induced H_2O_2 formation can also be detected at other sites as well. Exposure of the cells to the membrane-impermeable compound CeCl_3 in the presence of H_2O_2 results in the formation of an electron-dense precipitate, Ce(OH)_3OOH, whose presence can be detected by electron microscopy. When CeCl_3 is added to a cell system, detection of Ce(OH)_3OOH serves as evidence of the presence of extracellular H_2O_2 (41). When A549 cells were incubated with pyocyanin in the presence of extracellular CeCl_3, an electron-dense precipitate was observed at the plasma membrane (Fig. 2). We also observed ultrastructural changes in A549 cell mitochondria after 2 h of pyocyanin exposure, but only at the 100 μM concentration (Fig. 3).

Thus the combination of confocal and electron microscopy studies indicates that intracellular redox cycling of pyocyanin occurs in close proximity to the mitochondria. H_2O_2 production also occurs and can be detected outside the cell. Whether the H_2O_2 detected extracellularly arose as a consequence of pyocyanin redox cycling that occurred first at/near the mitochondria and then diffused cannot be determined from these studies.

**Pyocyanin depletes cellular ATP and alters mitochondrial 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. Consistent 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 O₂•− (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

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 $O_2^{-}$
intermediate and via the direct reduction of cyto-
Young (36). Because the reac-
tion 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 concentra-
tion-dependent decrease in ATP content relative to control. Values rep-
resent 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 bro-
ymide (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 mani-
fested 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 concentra-
tions 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 ac-
tivity. 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 MnSOD and CuZnSOD in the fractions confirmed that our fractionation system efficiently separated the mitochondrial 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 ~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 $\mathrm{O}_2^-\,$production leads to aconitase inhibition (14, 17), the evidence for this is indirect, and the intracellular site responsible for $\mathrm{O}_2^-\,$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 $\geq 30$), 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 nor 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).
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 DCFH₂ 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 DCFH₂ independently of ROS formation. Thus we cannot definitively attribute the detection of mitochondrial-associated DCF fluorescence to mitochondrial production of O₂⁻ and/or H₂O₂. 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-
tant effect 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.

![Fig. 10](image-url)

**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](image-url)

**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 CuZnSOD 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.

We thank Dr. Shankar Iyer for assistance with the HPLC determinations.

This work was supported in part by grants from the Research Service of the Department of Veterans Affairs (to B. E. Britigan, M. L. McCormick, and G. M. Denning), the Public Health Service (RO1 AI-43854, P30 DK-54759), and the Heartland Affiliate of the American Heart Association (to G. M. Denning, K. J. Reszka).

REFERENCES


