Oxidation of Thiols and Modification of Redox-Sensitive Signaling in Human Lung Epithelial Cells Exposed to Pseudomonas pyocyamin

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OXIDATION OF THIOLS AND MODIFICATION OF REDOX-SENSITIVE SIGNALING IN HUMAN LUNG EPITHELIAL CELLS EXPOSED TO Pseudomonas pyocyana

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The aim of this study was to examine the effects of pyocyanin exposure on mitochondrial GSH, other cellular thiols (thioredoxin-1, Trx-1), and oxidant-sensitive signaling pathways hypoxia inducible factor (HIF-1α) and heme oxygenase (HO-1) in A549 and HBE cell lines. A549 human type II alveolar epithelial cells and human bronchial epithelial (HBE) cells were treated with varying concentrations of pyocyanin extracted from Pseudomonas aeruginosa bacteria. Cytoplasmic and mitochondrial thiols and oxidant sensitive signal transduction proteins (HIF-1α and HO-1) were measured. Exposure to pyocyanin generated reactive oxygen species (ROS) in cellular mitochondria and altered total cellular glutathione (GSH). Pyocyanin, at concentrations present in conditions in vivo, increased oxidized Trx-1 in A549 human type II alveolar epithelial cells and HBE cells by 184 and 74%, respectively. Oxidized mitochondrial glutathione (GSSG) was elevated more than twofold in both cell types. Pyocyanin also increased the cellular oxidant-sensitive proteins HIF-1α and HO-1. Data indicate that pyocyanin-induced alterations in mitochondrial and cytosolic thiols, as well as oxidant-sensitive proteins, may contribute to P. aeruginosa-mediated lung injury.

Pseudomonas aeruginosa produces an acute necrotizing pneumonia with high frequency of mortality (Fick & Hata, 1989), and chronic lung infection in patients with cystic fibrosis (CF) or chronic bronchiectasis (Currie et al., 2003; Fick & Hata, 1989; Fick, 1989). Chronic lung injury is the primary cause of death in CF and is linked to coexistent P. aeruginosa infection (Currie et al., 2003; Fick & Hata, 1989; Fick, 1989). The mechanisms involved in P. aeruginosa-mediated tissue damage still remain unknown.

Pyocyanin is a redox-active compound produced by P. aeruginosa and has been detected in the sputum of patients with CF at concentrations up to 100 μM (Watson et al., 1986; Wilson et al., 1988). Pyocyanin undergoes cell-mediated aerobic redox cycling, resulting in reactive oxygen species (ROS) generation (Britigan et al., 1992; Cox, 1986), which is believed to contribute to lung damage (Lau et al., 2004; Schwarz et al., 2008). Cellular reducing equivalents such as NADH or NADPH are the predominant source of reducing equivalents that are transferred to pyocyanin, which then reduces oxygen to ROS, thereby placing the cells under increased oxidative stress (Hassan & Fridovich, 1980).

Most human cells possess several key mechanisms to limit damage produced by...
ROS. Among the major components of cellular antioxidant defenses are the thiol compounds (Dickinson & Forman, 2002; Meister & Anderson, 1983). Oxidant species such as hydrogen peroxide (H₂O₂) are removed by NADPH-dependent redox cycling of glutathione (GSH), catalyzed by glutathione peroxidase and glutathione reductase (Cross et al., 1987) (Figure 1). The GSH system, present in both cytoplasm and mitochondria, provides a majority of the H₂O₂ removing capacity in most cells (Cross et al., 1987).

A similar recycling of the thiol-containing peptide thioredoxin (approximately 14 kD), catalyzed by thioredoxin peroxidase and thioredoxin reductase, is also an important antioxidant system (Powis & Montfort, 2001; Arnér & Holmgren, 2000). Unique isoforms of the thioredoxin system components are found in the cytoplasm (thioredoxin-1, Trx-1), mitochondria (thioredoxin-2), and nucleus (Cadenas & Davies, 2000). This cycling of GSH and thioredoxin is an important mechanism for limiting exposure to and cytotoxicity from H₂O₂.

Reactive oxygen species (ROS) contribute to the regulation of key intracellular signaling mechanisms, and levels of intracellular antioxidants are regulated transcriptionally by ROS-modified intracellular signaling systems (D’Acquisto et al, 2002). Several pathways were suggested to be involved in ROS-regulated cell signaling, including the activation of different kinases and transcription factors such as hypoxia inducible factor (HIF-1α) and gene products such as heme oxygenase (HO-1) (Huang & Bunn, 2003). Both GSH and thioredoxin play important roles in regulating these redox-sensitive signaling pathways (McEligot et al., 2005).

Considering the fact that pyocyanin exposure leads to generation of intracellular ROS, it was postulated that pyocyanin might alter mitochondrial and cytosolic thiol systems and, subsequently, the oxidant-sensitive transcription factors and gene products regulated by them. The aim of this study was thus to determine the effects of pyocyanin on the mitochondrial and cytosolic thiol system and factors involved in this system.

**MATERIALS AND METHODS**

**Cells and Culture Conditions**

The human alveolar type II cell line A549 (CL-185; American Type Culture Collection, Rockville, MD) and the SV40-transformed normal human bronchial epithelial 16HBE14 cell line (Swords et al., 2002) (HBE, provided by Dr. Michael Apicella, Department of Microbiology, University of Iowa) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine
serum (FBS), 2 mM glutamine, and 500 μg/ml streptomycin.

**Pyocyanin Purification**

Pyocyanin was extracted from the broth culture of *P. aeruginosa* as previously described (Cox, 1986).

**Measurement of Glutathione (GSH)**

Following treatment with pyocyanin or vehicle, cells were harvested by scraping and centrifuged at 800 × g for 5 min. Glutathione disulfide (GSSG) and total glutathione (GSH + GSSG) were determined using a previously described spectrophotometric recycling assay (Griffith, 1980; Hook et al., 2009; Anderson, 1985). All biochemical determinations were normalized to protein content using the Bradford method.

**Mitochondrial Isolation**

HBE and A549 cell mitochondria were prepared by homogenization followed by differential centrifugation in isolation medium (250 mM sucrose, 5 mM HEPES, 0.1 M ethylenediamine tetraacetatic acid [EDTA], pH 7.2). To measure GSH content, mitochondria were collected, resuspended in 100 μl diethyleneetriame pentaacetic acid buffer, and GSH was quantified by the recycling assay (Griffith, 1980; Hook et al., 2009; Anderson, 1985). Immunoblot analysis of Mn superoxide dismutase (SOD) and CuZnSOD in the fractions confirmed that our observation system efficiently separated the mitochondrial and cytosolic fractions, respectively (data not shown).

**Thioredoxin Redox State**

This assay was performed as described before (Abdalla et al., 2005). Briefly, cells were collected by scraping and suspended in guanidine-Tris solution (6 M guanidine-HCl, 50 mM Tris, pH 8.3, 3 mM EDTA, and 0.5% Triton X100) supplemented with 50 mM iodoacetic acid (IAA), and incubated at 37°C for 30 min. Excess IAA was removed by Sephadex chromatography (MicroSpin G-25 columns, Amersham-Pharmacia). Samples were then run into native gel polyacrylamide electrophoresis and blotted to nitrocellulose paper (Watson et al., 2003). Primary antibody was anti-thioredoxin-1 (American Diagnostica, Greenwich, CT) and the secondary antibody conjugated to horseradish peroxidase (HRP; anti-goat immunoglobulin [lg] G–HRP conjugate, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were visualized using chemiluminescent detection (ECL, Amersham) using x-ray films (Kodak, Rochester, NY) (Watson et al., 2003; Holmgren & Fagerstedt, 1982; Das et al., 1997). Fully reduced control was included by adding 5 mM dithiothreitol (DTT).

**Immunoblot Analysis**

Cells were collected by scraping and centrifugation at 800 × g for 5 min. Cells were lysed by sonication in 10 mM Tris HCl (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The protein concentration was determined by the Bradford method and then used for the following immunoblot analyses:

*Heme oxygenase-1* Anti-HO-1 antibody was purchased from Stressgen, USA.

*HIF-1α* Portion of total cell lysate protein was separated on a 6.5% gradient Tris-HCl polyacrylamide gel, and blotted into PVDF membrane (Millipore Corporation, Bedford, MA). Blots were incubated with mouse anti-HIF-1 IgG (Pharmingen/Transduction Laboratories, San Diego, CA) and followed by secondary antibody, goat anti-mouse IgG (Pharmingen/Transduction Laboratories, San Diego, CA). Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL) and exposed to film. Relative measurements of immunoreactive proteins were obtained by densitometric scanning.

**Statistical Analysis**

All results are expressed as means ± standard error mean (SEM). For analysis limited to two groups, Student’s *t*-test was employed (*p* < .05).
FIGURE 2. Effect of pyocyanin on epithelial cell GSH and GSSG. (A) A549 or (B) HBE cells were incubated with indicated concentrations of pyocyanin or vehicle for 24 h, following which cellular GSH and GSSG levels were determined. Results shown are means ± SEM of cellular GSH, and GSSG, as a function of the concentration of pyocyanin employed (n = 3). Asterisk indicates significant difference from control (p < .05).

RESULTS

Influence of Pyocyanin on Epithelial Cell GSH and GSSG

Incubation of A549 cell monolayers with different concentrations of pyocyanin for 24 h resulted in a significant effect on GSH levels (Figure 2). Total GSH was significantly decreased by all concentrations of pyocyanin in A549 (Figure 2A) and HBE (Figure 2B) cells but not in a concentration-dependent manner. Pyocyanin exposure also resulted in a significant increase in GSSG in a non-concentration-related manner (Figure 2A). However, GSSG levels did not appear to be altered by pyocyanin in HBE cells (Figure 2B).

Pyocyanin Oxidizes Epithelial Cell Mitochondrial GSH

Subcellular fractions of A549 cells exposed to 25 μM pyocyanin, a concentration known to occur in vivo (Wilson et al., 1988), revealed that exposure to pyocyanin resulted in no significant changes in the total mitochondrial GSH levels (not shown) in A549 cells, but a significant increase approximately twofold in mitochondrial GSSG was noted (Figure 3A). A549 cells are a lung cancer cell line, and antioxidant defense systems may be different in malignant versus normal cells (Oberley & Buettner, 1979). In order to verify that the results obtained with A549 were applicable to nonmalignant epithelial cells, studies using monolayers of HBE cells were conducted. Exposure to pyocyanin significantly elevated approximately twofold oxidized mitochondrial GSH in HBE cells (Figure 3B).

Influence of Pyocyanin on Cellular Thioredoxin

Thioredoxin and GSH constitute major cellular thiol reducing systems. Our findings that pyocyanin exposure altered cellular GSH levels and increased levels of oxidized GSH in mitochondria led us to examine whether Trx-1 was also affected. As shown in Figure 4, pyocyanin exposure led to the oxidation of Trx-1 in both A549 and HBE cells. A pyocyanin
Pseudomonas pyocyanin-INDUCED OXIDATIVE STRESS

FIGURE 3. Influence of pyocyanin on oxidation of mitochondrial GSH in A549 cells and HBE. (A) A549 cells or (B) HBE cells were incubated with 25 μM pyocyanin or vehicle for 24 h, following which mitochondria were isolated by differential centrifugation. GSSG levels in mitochondria were determined and normalized to mitochondrial protein. Results shown are means ± SEM (n = 3). Asterisk indicates significant difference from control (p < .05).

FIGURE 4. (A) A549 and (B) HBE cells were treated with the indicated concentrations of pyocyanin for 24 h, and then partially and fully oxidized and reduced Trx-1 was detected by immunoblot. Results are representative of at least two separate experiments.

concentration-dependent increase in A549 and HBE cells oxidized Trx-1 levels was seen up to 184 and 74%, respectively. In control experiments, when DTT was added to the samples, only one band corresponding to reduced Trx-1 was seen (data not shown).

Pyocyanin Modulation of Redox-Regulated Transcription Factors

The findings that pyocyanin exposure increases oxidation of both cytosolic (Schwarzer et al., 2008) and mitochondrial (current work) GSH and Trx-1 suggest that pyocyanin exposure might exert an effect on redox-sensitive transcription factors. The effect of pyocyanin on redox-sensitive transcription factor (HIF-1) using immunoblot determinations of HIF-1α in whole-cell lysates of A549 and HBE cells. Pyocyanin at low concentrations decreased cellular HIF-1α protein below basal levels, while higher pyocyanin concentrations increased it at similar basal levels (Figure 5) in both A549 and HBE cells.

Pyocyanin Induces HO-1 Expression

Another cellular component shown to play a key role in protecting cells against oxidative stress is HO-1 (Ryter et al., 2006). As shown in Figure 6, a significant increase in HO-1 protein expression in both A549 and HBE cells exposed was noted.

DISCUSSION

Pyocyanin is one of several virulence factors secreted by P. aeruginosa (Lau et al., 2005). 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. Lau et al (2004) suggested mitochondria as one of the targets by which pyocyanin disrupts lung epithelial
cell functions and contributes to pathogenesis of lung injury resulting from both acute and chronic *P. aeruginosa* lung infection.

Results from confocal microscopy provide the most direct evidence to date that extracellularly administered pyocyanin reaches mitochondria, where it may enhance ROS generation (O’Malley et al., 2003). It is important to note that oxidation of dichlorofluorescein may potentially occur through mechanisms other than ROS production (O’Malley et al., 2003). Nevertheless, O’Malley et al. (2003) indicated that pyocyanin reaches mitochondria and subsequently redox cycles in or closely near cellular mitochondria.

Cellular GSH plays an important role in protecting the cell from oxidant-mediated injury and in modulating the action of redox-regulated transcription factors (Meister & Anderson, 1983). Cells placed under increased oxidative stress often exhibit an increase in their levels of GSSG (Fick & Hata, 1989). The biological importance of redox changes to mitochondrial thiols during oxidative stress

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#### FIGURE 5

The effect of different pyocyanin concentrations on HIF-1α levels after 6 h is shown using immunoblot determinations of HIF-1α in whole-cell lysates of (A) A549 and (B) HBE cells. Densitometric analyses of HIF-1α expression in A549 and HBE cells are also shown.

#### FIGURE 6

The effect of different pyocyanin concentrations on HO-1 protein levels at 24 h was assessed by immunoblot analysis in (A) A549 and (B) HBE cells. Densitometric analyses of HO-1 expression in A549 and HBE cells (n = 3, means ± SEM) are also illustrated. Asterisk indicates significant difference from control (p < .05).

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and cell death is well recognized (McEligot et al., 2005). Several investigators previously demonstrated that in vitro exposure of cells to pyocyanin resulted in alterations in cytosolic GSH (Schwarzer et al., 2008; Currie et al., 2003). Our current study also showed that the addition of pyocyanin to A549 cells resulted in a decrease in the total cellular GSH levels. Somewhat surprisingly, there was a significant increase in total cellular GSH levels in HBE cells, with no marked changes in GSSG levels, suggesting that there is a demand for synthesis of more reduced GSH as an indicative of oxidative stress (Figure 2B). However, direct measurement of the modification of mitochondrial GSH resulting from pyocyanin exposure has not been undertaken. Subcellular fractionation of epithelial cells exposed to 25 μM pyocyanin confirmed that pyocyanin exposure elevated mitochondrial GSSG.

Thioredoxin-1 is a multifunctional protein that has a redox-active disulfide at its active site. Tanaka et al. (2002) reported that Trx-1 functions both intracellularly and extracellularly as one of the key regulators of signaling in the cellular responses against oxidative stresses. Trx-1 translocates to the nucleus and increases the activity of several transcription factors in response to stress conditions (Janssen-Heininger et al., 2000). Both A549 and HBE cells exposed to pyocyanin showed greater oxidation of Trx-1 compared to control cells. Data suggest that ROS resulting from pyocyanin exposure is capable of oxidizing Trx-1.

Hypoxia-inducible factor-1α is a heterodimeric transcription factor that is constitutively expressed in a variety of cells under normoxic and hypoxic conditions (Huang & Bunn, 2003). Under hypoxia, HIF-1α is not degraded, and the stabilized protein heterodimerizes with HIF-1β. This results in DNA binding and transactivation of HIF-responsive genes (Huang & Bunn, 2003). Known inducers of HIF-1α expression in normoxic cells include bivalent metals, iron chelators, growth factors, and ROS (Huang & Bunn, 2003). Our data indicate that pyocyanin also enhanced the expression of HIF-1. This process may be mediated by pyocyanin-induced intracellular ROS generation involving GSH and Trx-1. Given our evidence that pyocyanin increases cellular levels of oxidized GSH and Trx-1, as well as the activity of HIF and probably other transcription factors, it seemed likely that pyocyanin may contribute to activation of HO-1. This was confirmed, as pyocyanin induced a rise in cellular HO-1 activity. These observations may be secondary to the effects of pyocyanin on cellular thiol systems. However, additional work is required to definitively link these events.

The ability of pyocyanin exposure to change the levels of oxidized GSH and Trx-1, as well as several transcription factors, may contribute to the pathogenesis of lung injury associated with P. aeruginosa infection. A direct link of the various events cannot be made with complete certainty based upon our data. Other investigators showed that pyocyanin operates through the Erk/p38 (MAPK) senescence pathway (Muller et al, 2009). Additional studies are necessary to more fully understand the possible involvement of other transcription factors, such as activator protein (AP)-1 and nuclear factor (NF)-κB, in response to pyocyanin. The effect of pyocyanin on cells may be quite varied depending upon the pyocyanin concentration to which they are exposed, the cell type involved and the relative level of endogenous antioxidant defenses.

REFERENCES


