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Mechanism for glutathione-mediated protection against the *Pseudomonas aeruginosa* redox toxin, pyocyanin

Michael Muller *, Neil D. Merrett

Department of Surgery, School of Medicine, University of Western Sydney, Building 30, Goldsmith Avenue, Campbelltown, NSW 2560, Australia

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ABSTRACT

Pseudomonas aeruginosa is an important human pathogen associated with several acute and chronic conditions, including diseases of the airways and wounds. The organism produces pyocyanin, an extracellular redox toxin that induces oxidative stress, depletes intracellular glutathione (GSH) and induces proliferative arrest and apoptosis, thus compromising the ability of tissue to repair itself. GSH is an important intra- and extracellular antioxidant, redox buffer and detoxifies xenobiotics by increasing their polarity, which facilitates their elimination. As previous studies have reported exogenous GSH to be protective against pyocyanin toxicity, this study was undertaken to explore the mechanism by which GSH protects host cells from the deleterious effects of the toxin. Co-incubation of pyocyanin with GSH resulted in a time-dependent diminished recovery of the toxin from the incubation medium. Concurrently, a highly polar green-colored metabolite was recovered that exhibited a UV–visible spectrum similar to pyocyanin and which was determined by mass spectrometry to have a major ion (m/z = 516) consistent with a glutathione conjugate. The ability of the conjugate to oxidize NADPH and to reduce molecular oxygen with the production of reactive oxygen species was comparable to pyocyanin yet it no longer demonstrated cytotoxicity towards host cells. These data suggest that GSH forms a cell-impermeant conjugate with pyocyanin and that availability of the thiol may be critical to minimizing the toxicity of this important bacterial virulence factor at infection sites. Our data indicate that for GSH to have a clinically effective role in neutralizing pyocyanin, the thiol needs to be available at millimolar concentrations.

1. Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen frequently encountered in clinical situations. The bacterium is a major cause of acute ventilator-associated pneumonia [1] and colonizes the airways of patients with chronic respiratory diseases, including those with cystic fibrosis or bronchiectasis, forming persistent biofilm communities in the airways [2] that are recalcitrant to antibiotic therapy and result in progressive pulmonary dysfunction and, ultimately, respiratory failure. In addition, *P. aeruginosa* continues to be a significant cause of infection for burns patients [3] while more recently the organism has been identified as a frequent colonizer of chronic dermal lesions and an important determinant in their failure to heal [4,5]. Moreover, a strong negative association between pseudomonal infection and skin graft outcomes has been reported [6].

P. aeruginosa possesses a substantial armamentarium of virulence factors including pyocyanin (Fig. 1), a blue redox active phenazine derivative that it secretes in considerable quantities. A substantial and growing body of evidence indicates pyocyanin plays a major role in the pathology of pseudomonal infections. The toxin has been identified as a critical component in the successful establishment of lung infections in mice [7] and is a major contributor to the lung pathology associated with cystic fibrosis [8]. A potent inhibitor of cellular replication, pyocyanin induces premature cellular senescence [9] and directly contributes to impaired wound healing [10]. The toxicity of pyocyanin is due to its ability to readily penetrate cell membranes and undergo redox cycling using molecular oxygen as an electron acceptor resulting in the generation of the reactive oxygen species (ROS), particularly hydrogen peroxide, with consequent induction of oxidative stress and disruption of intracellular redox homeostasis [9,11–13].

Glutathione (GSH) is a ubiquitous thiol present in mammalian cells that can reach millimolar concentrations and is actively exported to the interstitial fluids of various physiologic compartments. It is the major endogenous antioxidant and redox buffer for maintaining cellular redox homeostasis and is extensively

* Corresponding author at: Department of Surgery, School of Medicine, University of Western Sydney, Locked Bag 1797, Penrith, NSW 2751, Australia. Tel.: +61 2 4620 3739; fax: +61 2 4620 3890.
E-mail address: m.muller@uws.edu.au (M. Muller).

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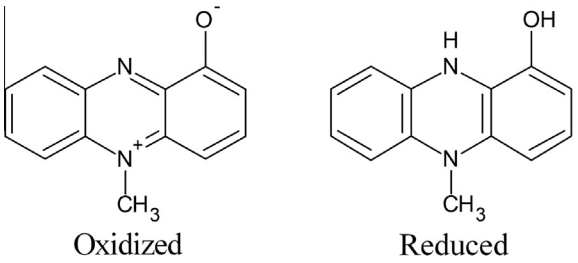

Fig. 1. Structures for oxidized and reduced forms of pyocyanin.

involved in the detoxification and metabolism of xenobiotics compounds [14]. Conjugation with GSH, either directly (non-enzymatic) or by glutathione-*S*-transferase mediated reactions, detoxifies a large range of compounds by increasing their aqueous solubility, thus aiding in their elimination. The effectiveness of the GSH detoxification system depends on maintaining adequate intracellular levels of GSH. However, studies of normal host cells have demonstrated that sub-lethal concentrations of pyocyanin, that are typically found at infection sites [10], alter the intracellular redox environment to that of a more oxidized state due to *S*-glutathionylation reactions resulting in the formation of mixed protein disulfides [12] with consequent depletion of GSH levels [12,13].

The bioavailability of physiologic concentrations of exogenous GSH plays an important role in minimizing the in vitro cytotoxicity of pyocyanin [9,15]. However, although many cells export GSH to the extracellular space they are unable to import the thiol directly into the cell. Consequently, host cells must rely on de novo biosynthesis of GSH from readily available precursors, such as cysteine [16]. This suggests that for exogenously supplied GSH to have an antioxidant effect on pyocyanin-generated ROS, those species must be present in the extracellular space. A previous study using catalase, which is also cell impermeant, indicated much of the pyocyanin-induced ROS are due to the extracellular reduction of molecular oxygen [12]. The protective effect of exogenous GSH [9,15] supports this hypothesis. However, the non-enzymatic reaction of GSH with hydrogen peroxide is relatively slow compared to glutathione peroxidase- or catalase-mediated reactions [17] suggesting that other mechanisms may be involved in GSH-mediated protection against pyocyanin.

GSH readily undergoes Michael-type addition reactions with substituted aromatic compounds [18] and, based on its structure, it may be expected such a reaction may occur with pyocyanin. Indeed, during the course of previous investigations [15], it was observed that pyocyanin underwent a color change from blue to green in the presence of exogenous GSH, which could be detected in phenol red-free medium. Similar color changes were not noted when alternative antioxidants, such as ascorbate or polyphenols were employed [19]. It was therefore postulated that the protective role of GSH may be due, in part, to its conjugation with pyocyanin resulting in modification of the toxin's biological activity.

Here, evidence is presented to demonstrate that incubation of pyocyanin in the presence of GSH results in the formation of a novel metabolite, glutathionyl-pyocyanin. The cell-free synthesis, purification, redox and biological activities of the metabolite are described.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma–Aldrich (Sydney, Australia) unless otherwise indicated.

2.2. Synthesis and quantitation of pyocyanin

Pyocyanin was prepared according to the method of Knight et al. (1979) [20] and purified by high performance thin layer chromatography (HPTLC; Merck Silica-Gel 60) as previously described [15]. The concentration of pyocyanin was determined by spectrophotometry in methanol at 318 and 690 nm using molar extinction coefficients of 30,200 and 5816, respectively while its identity was confirmed by mass spectrometry with $m/z = 211.1$. The purified compound was reconstituted in methanol, stored at −80 °C and protected from light at all times. The concentrations of pyocyanin used in the following experiments are comparable to those identified in wound exudates of infected patients [10].

2.3. Tissue culture

Normal human dermal fibroblasts (HDFs) were isolated, after obtaining informed consent, from biopsies of excess healthy skin tissue obtained from patients undergoing reconstructive surgery at the Concord Repatriation General Hospital (Concord, NSW), in accordance with the approval of the Sydney South West Concord Repatriation General Hospital Ethics and Research Committee. HDFs were grown on collagen-coated tissue culture-ware and maintained in complete Minimal Essential Medium (MEM; Invitrogen, Sydney) containing 5% fetal bovine serum (FBS, Interpath, Sydney) and glutamine (2 mM). Experiments were performed using HDFs between passages 3 and 8.

2.4. Recovery of pyocyanin from culture media

HDFs were grown to confluence in 12-well tissue culture plates. The recovery of pyocyanin (25 μM) from wells containing 1 ml of cell-free MEM, MEM plus HDFs and cell-free MEM supplemented with GSH (10 mM) after incubation at 37 °C for 24 h under aerobic conditions (5% CO_2) was determined by 3 sequential extractions with equal volumes of chloroform, pooling the organic extracts and evaporating to dryness under a stream of nitrogen. The residue was reconstituted in methanol (1 ml) and the pyocyanin concentration determined by spectrophotometry at 690 nm.

2.5. Cell-free synthesis and purification of a pyocyanin metabolite

Pyocyanin (25 μM) was incubated under anaerobic conditions in acid washed sealed glass vials with GSH (0.1 or 1 mM) in phosphate buffer (KH_2PO_4, 125 mM; pH 7.4) and in the dark at 37 °C for periods up to 6 h. Control samples consisting of pyocyanin alone in phosphate buffer under identical conditions were included. Aseptic techniques were employed at all times and all solutions were sterile filtered using 0.22 μm filters prior to use to exclude microbial contamination and subsequent metabolism of pyocyanin. Anaerobic conditions were used to avoid artifacts associated with the autoxidation of GSH under aerobic conditions and the effectiveness of the de-oxygenation procedure, which consisted of nitrogen purging of all solvents and containers, verified by sensitive oxygen membrane polarographic detection (Rank Brothers, England).

Following the incubation period unreacted pyocyanin was extracted by adding 1 volume of water (to assist phase separation) and 1 volume of chloroform to 1 volume of the aqueous medium and vortexing. The organic phase was carefully removed to avoid phase contamination and the chloroform extraction step repeated twice more. Extraction of the reaction mixture with chloroform resulted in unreacted pyocyanin partitioning into the lower organic phase while a green material remained in the upper aqueous phase. The organic extracts were combined and dried