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Original Contribution

Polyphenol cytotoxicity induced by the bacterial toxin pyocyanin: role of NQO1

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ABSTRACT

Pyocyanin is an important bacterial redox-active toxin produced by the opportunistic human pathogen *Pseudomonas aeruginosa*. The bacterium is a cause of serious infections of the respiratory tract, particularly for those with cystic fibrosis and for those with burn injuries. Pyocyanin induces oxidative stress and causes cells to become prematurely senescent, which compromises tissue remodeling and wound repair. A diverse range of antioxidants have been found useful in preventing oxidant-induced cellular senescence, including quercetin, a common dietary polyphenol. This study evaluated the effectiveness of three common polyphenols (quercetin, (+)-catechin, and (−)-epicatechin) as potential inhibitors of pyocyanin-induced senescence. Whereas at the lowest concentration the polyphenols maintained cellular replicative capacity, in the presence of pyocyanin they unexpectedly displayed concentration-dependent cytotoxicity with a rank order of quercetin>epicatechin>>catechin. On oxidation, polyphenols with B-ring catechol functionality form toxic alkylating quinones that are normally inactivated by cellular antioxidant defense and redox maintenance systems, including reduction by ascorbate and NAD(P)H:quinone oxidoreductase 1 (NQO1). Pyocyanin inhibited cellular NQO1 activity at low micromolar concentrations, but the presence of exogenous ascorbate eliminated pyocyanin-induced polyphenol cytotoxicity. These data indicate that pyocyanin compromises cellular redox maintenance systems, leaving cells susceptible to the adverse effects of otherwise nontoxic redox-active compounds.

Pseudomonas aeruginosa is a common opportunistic human pathogen that exploits compromised host defenses. The bacterium is a cause of severe acute and chronic infections of the airways and lungs. In addition, patients with burn injuries are particularly susceptible to infection by the organism and, in many cases, the infections are difficult to eradicate owing to compromised circulation and antibiotic resistance. Pyocyanin is an important virulence factor produced by the bacterium that is able to further compromise host defenses [1] and is considered to be a critical factor in lung infection [2], with its production being up regulated in response to host immune activation [3]. Pyocyanin induces many diverse effects in mammalian host cells owing, in part, to its ability to undergo redox cycling, with disturbances to intracellular redox status and the generation of reactive oxygen species [4]. However, it is the persistent low-level production of hydrogen peroxide that enables pyocyanin to induce cell cycle arrest and premature senescence in host cells, resulting in compromised tissue repair [5,6]. Cellular proliferation is a critical component of the host tissue repair mechanism, and factors that inhibit that process result in delayed healing, fibrosis, scar tissue formation, and increased risk of further infection [7].

Cellular senescence is rapidly emerging as an important mechanism involved in several pathological conditions [8,9], including chronic lung disease [10]. Various factors induce cells to become senescent, including extensive rounds of cell replication leading to telomere attrition [11] and oxidative stress [12]. On becoming senescent, cells of various lineages display arrested cell cycle progression and altered gene expression and exhibit a more proinflammatory phenotype compared to their quiescent counterparts [13]. These changes are thought to disturb normal tissue homeostasis and promote microenvironmental changes consistent with an aberrant wound repair response [8,9].

Several antioxidants have been found useful in preventing oxidant-induced cell cycle arrest and development of the senescent phenotype, including glutathione (GSH) [5] and quercetin [14,15], a common dietary polyphenol. Polyphenols are a diverse group of compounds found in fruits and vegetables and considered beneficial to human health when consumed regularly as part of a balanced diet [16]. In vitro, they possess a broad spectrum of biological activities that include antioxidant [17], anti-inflammatory [18], and antimicrobial properties [19]. Recent evidence suggests they improve tissue remodeling and wound repair in vivo [20]. Owing to their relative safety and beneficial biological effects polyphenols have been proposed for use in several clinical conditions [15,21,22], including those in which *P. aeruginosa* may be present [23,24].

Although it was previously demonstrated that the use of exogenous GSH was effective in protecting cells from the senescence-inducing effect of pyocyanin [5], the toxin rapidly depletes GSH owing to redox reactions [4]. In the quest for more stable compounds to inhibit pyocyanin-induced senescence and to facilitate the tissue repair process within a clinical setting, the polyphenols, with their

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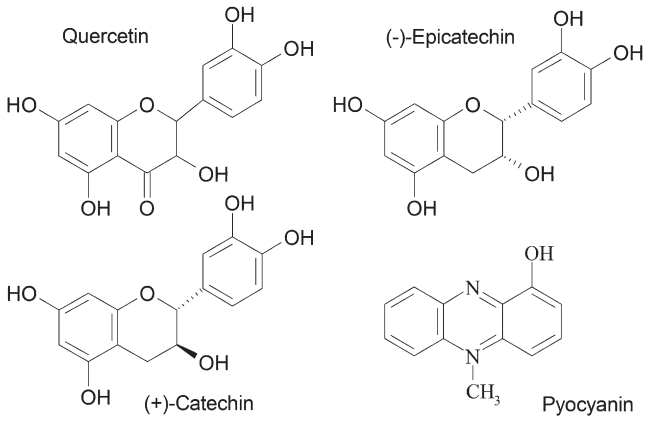

Fig. 1. Chemical structures of quercetin, (+)-catechin, (−)-epicatechin, and pyocyanin.

negligible in vivo toxicity and their antioxidant properties, seemed promising candidates. This study examined the ability of three common dietary polyphenols (quercetin, (+)-catechin, and (−)-epicatechin; Fig. 1) to protect proliferating cells from the senescence-inducing effect of pyocyanin. Unexpectedly, although capable of maintaining cellular replicative competency at low concentrations, each of the polyphenols exhibited concentration-dependent cytotoxicity in the presence of the bacterial toxin but not in its absence. It was found that pyocyanin is a potent inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase; EC 1.6.99.2) and that supplemental ascorbate was protective within this setting. These findings have important clinical implications for the use of polyphenols when pseudomonal infections may be involved. The data further suggest that individuals with NQO1 polymorphisms may be susceptible to polyphenol toxicity and that the safety of polyphenol supplements, in general, warrants further investigation.

Materials and methods

Reagents

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

Cell culture

Human lung-derived epithelial-like (A549) cells from the ATCC were used because the effects of pyocyanin [5] and polyphenols [25] on these cells individually have been well characterized. Cells were grown and maintained in complete RPMI 1640 medium (Invitrogen, Sydney, Australia) containing 10% fetal bovine serum (Invitrogen), glutamine (2 mM), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. All experiments were conducted in complete RPMI unless otherwise indicated.

Preparation of pyocyanin

Pyocyanin was prepared and purified by thin-layer chromatography as described previously [26]. The purified pigment was stored at −80 °C in methanol and protected from light at all times. Before use, the methanol was stripped off in a stream of nitrogen gas and the dried pyocyanin reconstituted in complete RPMI medium.

Preparation of polyphenols

For all experiments, the polyphenols were initially dissolved in dimethyl sulfoxide (DMSO) before being diluted to the required concentration in the appropriate working medium. All controls were similarly prepared with DMSO. The concentration of DMSO did not exceed 0.05% for any experiment.

Determination of cellular proliferation and viability

The proliferative ability of the cells, assessed as population doublings, was determined by cell counts using the trypan blue assay as described [5]. Cells were pretreated with polyphenols 30 min before the addition of pyocyanin.

Cell viability was determined using the CellTiter-Glo luminescence cell viability assay kit (Promega, Sydney, Australia). Cells were plated at 1×10^4 cells/ml (100 μl/well) in 96-well tissue culture plates in complete RPMI. After incubation for 24 h the medium was removed and fresh complete medium containing the appropriate concentration of polyphenol (quercetin, (+)-catechin, or (−)-epicatechin) was added. The cells were incubated for 30 min before pyocyanin (final concentration 10 μM) or pyocyanin-free vehicle was added, after which the cells were incubated for a further 24 h. The luminescent signal was detected using a FluorStar Optima plate reader (BMG Labtech, Australia) equipped with a luminescence probe.

Plasma membrane integrity assay

The integrity of the plasma membrane was assessed by determining leakage of cellular proteases using the MultiTox-Fluor Multiplex cytotoxicity assay kit (Promega). Cells were plated at 1×10^4 cells/ml (100 μl/well) into clear-bottom opaque-wall 96-well tissue culture plates and incubated for 24 h after which the medium was removed and fresh complete medium containing the appropriate concentration of polyphenol (quercetin, (+)-catechin, or (−)-epicatechin) was added. The cells were incubated for 30 min before pyocyanin (final concentration 10 μM) was added and then incubated for a further 24 h. This assay makes use of the principle that protease leakage from damaged cells hydrolyzes a nonfluorescent, cell-impermeative peptide (bis-alanylalanylphenylalanyl-rhodamine 110; bis-AAF-R110) to fluorescent rhodamine 110 (R110). Fluorescence due to R110 formation was detected with a FluorStar Optima plate reader set at 485/520 nm ($\lambda_{em}/\lambda_{ex}$).

Annexin V binding assay

The expression of phosphatidylserine on the outer leaflet of the plasma membrane, indicative of the loss of membrane asymmetry, was assessed by determining annexin V binding. Cells were plated at

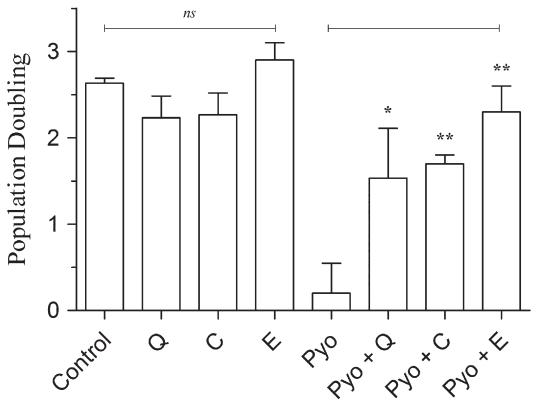

Fig. 2. Polyphenols inhibit replicative arrest induced by pyocyanin. Treatment of cells (1×10^4 cells/ml in six-well plates) with the polyphenol quercetin (Q), catechin (C), or epicatechin (E) for 24 h did not result in a significant loss of population doubling potential when used at a concentration of 25 μM. In contrast, when used alone, pyocyanin (10 μM) arrested cell proliferation compared to the untreated control. Preincubation of cells with the polyphenols (25 μM) 30 min before the addition of pyocyanin (10 μM) resulted in a statistically significant increase in population doublings compared to pyocyanin alone. Results represent the means ± SD, $n=3$ (*ns*, not significant; *$p<0.01$; **$p<0.001$).