Generation of Reactive Oxygen Species Mediated by 1-Hydroxyphenazine, a Virulence Factor of Pseudomonas aeruginosa

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*Supporting Information

ABSTRACT: 1-Hydroxyphenazine (1-HP) is a virulence factor produced by Pseudomonas aeruginosa. In this study, supercoiled plasmid DNA was employed as an analytical tool for the detection of ROS generation mediated by 1-HP. These assays provided evidence that 1-HP, in conjunction with NADPH alone or NADPH and the enzyme NADPH:cytochrome P450 reductase, mediated the production of superoxide radical under physiological conditions. Experiments with murine macrophage RAW264.7 cells and profluorescent ROS probes dichlorodihydrofluorescein or dihydroethidine provided preliminary evidence that 1-HP mediates the generation of intracellular oxidants. Generation of reactive oxygen species may contribute to the virulence properties of 1-HP in P. aeruginosa infections.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that infects the lungs of immunocompromised patients and individuals with cystic fibrosis.1 This organism produces a number of virulence factors, including the phenazine derivatives pyocyanin, 1-phenazine carboxylic acid, and 1-hydroxyphenazine (1-HP) (Chart 1).2 The best characterized of these is pyocyanin.3,4 Pyocyanin contributes to P. aeruginosa pathogenicity and tissue damage in the host by mechanisms involving the production of reactive oxygen species (ROS): superoxide radical, hydrogen peroxide, and hydroxyl radical.3−5 Pyocyanin generates superoxide radical via redox cycling. This process is initiated via direct (nonenzymatic) reduction of pyocyanin by NADPH, NADH, and, possibly, the cellular thiol glutathione.6−10 Subsequent reactions of one- or two-electron-reduced pyocyanin intermediates with molecular oxygen generate superoxide radical anion (O2•−).6−9 Superoxide radical, in turn, gives rise to hydrogen peroxide and hydroxyl radical by the well-known cascade of reactions shown in (unbalanced) eq 1, involving disproportionation of superoxide to yield hydrogen peroxide followed by metal-mediated Fenton-type reactions that produce hydroxyl radical.11,12

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\text{O}_2 \rightarrow \text{O}_2^{•−} \rightarrow \text{H}_2\text{O}_2 + \text{M}^{n+} \rightarrow \text{HO}^{•} + \text{M}^{(n+1)+}
\] (1)

Although the phenazine virulence factors of P. aeruginosa often are discussed as a group, their molecular structures are distinct and they should be expected to possess different chemical and biological properties. For example, the presence of a methylated phenazine nitrogen in pyocyanin drastically alters its redox potential relative to 1-HP (making pyocyanin...
Figure 1. DNA strand cleavage by 1-HP driven by NADPH and NADPH plus NADPH:cytochrome P450 reductase (CYPOR). Superoiled plasmid DNA (1 μg) was incubated with 1-HP (1-HP, 100 μM) and the indicated concentration of NADPH (200 μM, 400 μM, 750 μM, 1 mM) in sodium phosphate buffer (50 mM, pH 7.0) at 24 °C for 12 h. Some assays contained CYPOR (0.05 U/mL) or catalase (Cat, 100 μg/mL) and desferal (Des, 1 mM) as indicated. Yields of DNA strand breaks in each reaction (S) were measured by agarose gel electrophoresis and calculated using the equation: S = −ln f₀, where f₀ is the fraction of plasmid remaining as supercoiled form I. Lane 3, S = 0.29 ± 0.16 above background; lane 4, S = 1.02 ± 0.39 above background; lane 7, S = 0.63 ± 0.10 above background; lane 8, S = 1.52 ± 0.27 above background; lane 11, S = 0.82 ± 0.19 above background; lane 12, S = 1.61 ± 0.58; lane 15, S = 0.91 ± 0.13 above background; lane 16, S = 1.83 ± 0.44 above background.

Materials and Methods. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Fisher Scientific (Fairlawn, NJ). 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) was from Oxis International, Inc. (Portland, OR), 2,7-Diamino-10-ethyl-9-fluorescein diacetate (H₂DCFDA) were from Invitrogen (Carlsbad, CA), respectively. Other reagents were of the highest purity available and were obtained from the following suppliers and used without purification: sodium phosphate, NADPH, acetonitrile, desferal, NADPH:cytochrome P450 reductase (CYPOR), catalase, and superoxide dismutase (SOD) were from Sigma-Aldrich Chemical Co. (St. Louis, MO); agarose was from Seakem; HPLC grade solvents methanol and ethanol were from Fisher Scientific (Pittsburgh, PA); and ethidium bromide was from Roche Molecular Biochemicals (Indianapolis, IN). Plasmid DNA pGL2BASIC was prepared using standard protocols. 1-HP was prepared using a published procedure. 31

Cleavage of Plasmid DNA by 1-HP. In a typical DNA cleavage assay, supercoiled plasmid DNA (pGL2BASIC, 1 μg) was incubated with 1-HP (100 μM) and NADPH (200 μM) in sodium phosphate buffer (50 mM, pH 7.0), in a final volume of 40 μL. Some assays contained NADPH:cytochrome P450 reductase (0.05 U/mL, where one unit is defined as the amount of enzyme required to cause the reduction of 1 μmol of cytochrome c by NADPH per min at pH 7.4 at 37 °C), SOD (100 μg/mL), catalase (100 μg/mL), desferal (1 mM), and methanol (1 mM) or ethanol (1 mM). Heat-denatured catalase and SOD for control reactions were prepared by heating aliquots of the enzyme stock solutions in a boiling water bath for 4 h. Reactions were initiated by addition of NADPH or NADPH:cytochrome P450 reductase, wrapped with aluminum foil to prevent exposure to light, and incubated under air at 24 °C for 12 h. Following incubation, the reactions were mixed with 50% glycerol loading buffer (6.6 μL), and the resulting reaction mixture was loaded onto a 0.9% agarose gel. The gel was electrophoresed for approximately 2 h at 90 V in TAE buffer and then stained in a solution of ethidium bromide (0.3 μg/mL) for 3 h. DNA in the gel was visualized by UV-transillumination, and the amount of DNA in each band was quantified using an Alpha Innotech IS-1000 digital imaging system. Yields of DNA strand breaks in each reaction (S) were calculated using the equation: S = −ln f₀, where f₀ is the fraction of plasmid remaining as supercoiled form I.

Nitro Blue Tetrazolium Assay. In a typical assay, 1-HP (100 μM), NADPH (500 μM), nitro blue tetrazolium chloride (500 μM), and NADPH:cytochrome P450 reductase (0.13 U/mL) were incubated in sodium phosphate buffer (50 mM, pH 7, 200 μL) at 24 °C under aerobic conditions for 12 h. After incubation, solid sodium carbonate was added to the assay to adjust the pH to 10. The assay was diluted with water to a final volume of 1 mL, and the absorbance at 560 nm was measured. Two control experiments were performed using the same conditions except lacking 1-HP or NADPH, respectively.

Cell Culture and Imaging of DHE and DCF Fluorescence. Murine macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, VA) was routinely cultured in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL streptomycin, as described previously. 24 For the experiments, cells were seeded into 4-well Permanox slides (Nalge Nunc International Corp., Naperville, IL) to reach 90% confluency overnight. Cells were washed and incubated in DMEM containing 0.5% FBS (0.5 mL/well) with 1-HP or DMNQ at the indicated concentrations for 30 min. Then, 20 μM DHE or H₂DCFDA was added, and confocal fluorescence microscopy was performed. 25,26 Images were acquired from three or more independent fields/well and were processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems). The levels of fluorescence were averaged using SimplePCI software (Compix, Cranberry Township, PA).

Results and Discussion. Evidence That 1-HP, in Conjunction with NADPH or NADPH and NADPH:cytochrome P450 reductase, Mediates ROS Generation. In this study, we employed supercoiled plasmid DNA as an analytical tool for the detection of ROS. This assay capitalizes on the fact that oxidative DNA strand cleavage causes conversion of the supercoiled plasmid to the open circular form. 27–30 These two forms of plasmid DNA are easily separated and quantitatively measured using agarose gel electrophoresis, staining the DNA with ethidium bromide, and digital image analysis of the stained gel. In general, intracellular generation of superoxide radical causes a host of deleterious effects including oxidative damage to DNA, proteins, and lipids and cytotoxicity. 3,7,12,21–24 In the context of the plasmid assay used here, it is important to note that
hydroxyl radical, or a species of similar reactivity, is the actual agent responsible for DNA strand cleavage induced by superoxide radical.\textsuperscript{12,36} Nonetheless, the use of additives such as superoxide dismutase, catalase, trace metal chelators, and hydroxyl radical scavenging agents can reveal whether the $\text{O}_2^{-*}$ \rightarrow $\text{H}_2\text{O}_2$ \rightarrow $\text{HO}^*$ reaction cascade is operative.\textsuperscript{12,16,37} There are a number of examples in which enzymatic reduction drives redox cycling of small organic molecules.\textsuperscript{16,38,39} Accordingly, we examined the effect of NADPH:cytochrome P450 reductase (CYPOR) on the production of ROS by 1-HP. This enzyme has been implicated in the redox cycling of a wide variety of compounds.\textsuperscript{16,38–43}

We found that 1-HP (100 $\mu$M) in conjunction with NADPH (100 $\mu$M) in sodium phosphate buffer (50 mM, pH 7) mediated the formation of direct strand breaks in the supercoiled plasmid DNA substrate (Figure 1). The amount of strand cleavage increased with increasing concentrations of NADPH. At lower concentrations of NADPH (e.g., 100 $\mu$M), the yields of strand cleavage induced by 1-HP (100 $\mu$M) were enhanced 3.5-fold by the presence of CYPOR. Control assays showed that 1-HP alone or NADPH and CYPOR without 1-HP did not yield substantial amounts of strand cleavage.

In general, the generation of direct strand breaks (as opposed to base-labile lesions) caused by 1-HP in the plasmid assay was consistent with radical-mediated strand cleavage.\textsuperscript{36} Therefore, we set out to characterize the nature of the DNA-cleaving radicals generated by 1-HP. We found that addition of classical hydroxyl radical scavengers\textsuperscript{12} methanol or ethanol (1 mM) inhibited the strand cleavage process (Figure 2). Addition of the metal chelator desferal inhibits strand cleavage, consistent with the involvement of adventitious transition metals in a Fenton-type generation of hydroxyl radical from $\text{H}_2\text{O}_2$ (Figure 2).\textsuperscript{12} Similarly, the hydrogen peroxide-destroying enzyme catalase inhibited the strand cleavage process. A control reaction showed that heat-denatured catalase did not significantly inhibit the strand cleavage process. In fact, heat-denatured catalase slightly increased strand cleavage yields, probably due to the release of redox-active transition metals that can participate in Fenton-type reactions; see Figure S1.) Addition of the enzyme superoxide dismutase (SOD) had a small inhibitory effect on the strand cleavage process. SOD merely accelerates the already fast disproportionation of superoxide to $\text{H}_2\text{O}_2$ and $\text{O}_2$ and therefore may not be expected to have a significant effect on strand cleavage that arises from the production of superoxide radical.\textsuperscript{12} Heat-denatured SOD exerted little effect on the reactions. In cases where SOD inhibits ROS-mediated strand cleavage, it may reflect a role for superoxide in the reduction of transition metals required for the Fenton reaction.\textsuperscript{12,44}

The yields of strand cleavage increased as a function of both 1-HP and NADPH concentrations (Figures 3 and 4). A side-by-side comparison showed that 1-HP (0.28 ± 0.02 breaks above background) generated approximately 0.28 times as many direct strand breaks as did the archetypal\textsuperscript{45,46} redox-cycling agent 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, 0.97 ± 0.13 breaks above background) when each agent (100 $\mu$M) was incubated in sodium phosphate buffer (50 mM, pH 7) at 24 °C for 12 h. Yields of DNA strand breaks in each reaction were measured by agarose gel electrophoresis and calculated using the equation $S = -\ln f_1$, where $f_1$ is the fraction of plasmid remaining as supercoiled form I.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Evidence that DNA strand cleavage by 1-HP involves $\text{O}_2^{-*}$ \rightarrow $\text{H}_2\text{O}_2$ \rightarrow $\text{HO}^*$. Supercoiled plasmid DNA (1 $\mu$g) was incubated with 1-HP (100 $\mu$M), NADPH (200 $\mu$M), and CYPOR (0.05 U/mL) in sodium phosphate buffer (50 mM, pH 7.0) at 24 °C for 12 h. Additive concentrations were as follows: catalase and SOD (100 $\mu$g/mL), desferal (1 mM), methanol (1 mM), and ethanol (1 mM). Yields of DNA strand breaks in each reaction were measured by agarose gel electrophoresis and calculated using the equation $S = -\ln f_1$, where $f_1$ is the fraction of plasmid remaining as supercoiled form I.

**Figure 3.** Dependence of strand cleavage yields on the concentration of 1-HP. Supercoiled plasmid DNA (1 $\mu$g) was incubated with 1-HP (100 $\mu$M), NADPH (200 $\mu$M), and CYPOR (0.05 U/mL) in sodium phosphate buffer (50 mM, pH 7.0) at 24 °C for 12 h. This plot is derived from the experiments illustrated in Figure 1. Yields of DNA strand breaks in each reaction were measured by agarose gel electrophoresis and calculated using the equation $S = -\ln f_1$, where $f_1$ is the fraction of plasmid remaining as supercoiled form I.

**Figure 4.** Dependence of strand cleavage yields on NADPH concentration. Supercoiled plasmid DNA (1 $\mu$g) was incubated with 1-HP (100 $\mu$M), varying concentrations of NADPH (200–1000 $\mu$M), and CYPOR (0.05 U/mL) in sodium phosphate buffer (50 mM, pH 7.0) at 24 °C for 12 h. This plot is derived from the experiments illustrated in Figure 1. Yields of DNA strand breaks in each reaction were measured by agarose gel electrophoresis and calculated using the equation $S = -\ln f_1$, where $f_1$ is the fraction of plasmid remaining as supercoiled form I.
spectrophotometric assay that detected the conversion of nitro blue tetrazolium to monoformazan. In this experiment, 1-HP (200 μM) and nitro blue tetrazolium chloride (500 μM) were incubated with NADPH (500 μM) and CYPOR (0.13 U/mL) in sodium phosphate buffer (50 mM, pH 7) at 24 °C for 12 h. In order to detect monoformazan generated in the assay, the pH of the mixture was adjusted to 10 prior to measuring the absorbance at 560 nm. In an assay containing 1-HP, CYPOR, and NADPH, we observed a 3.4-fold increase in absorbance at 560 nm versus a control sample containing only CYPOR and NADPH and a 9.7-fold increase versus a control containing the CYPOR enzyme without the NADPH substrate.

We also employed cytochrome c as a probe for superoxide radical production. Reduction of the heme unit in cytochrome c

Figure 5. Evidence that 1-HP generates ROS in cells. Murine macrophage RAW264.7 cells were incubated with 1-HP (5−30 μM) or DMNQ (30 μM) for 30 min; then, profluorescent ROS probes H2DCFDA (20 μM) or DHE (20 μM) was added, and confocal microscopy was performed. (A, C) Confocal microscopic images of cells treated with 1-HP or DMNQ, respectively. (B, D) Bar graphs showing the increase in fluorescence from images acquired from three or more independent fields.
by superoxide radical anion generates an absorbance increase at 550 nm.49 We found that incubation of 1-HP (50 μM), NADPH (100 μM), CYPOR (0.05 U/mL), and cytochrome c (100 μM) for 12 h at 24 °C gave an absorbance increase at 550 nm above background (cytochrome c in buffer) that, based upon the known difference in molar absorptivity of reduced minus oxidized cytochrome c of 21 000 M−1 cm−1, corresponded to the generation of 1 equiv of superoxide radical based on 1-HP. The signal was substantially diminished in control samples containing SOD, but it was not significantly affected by the presence of heat-denatured SOD. Control experiments showed that 1-HP alone, NADPH alone, or CYPOR alone did not generate significant levels of superoxide radical (Figure S2).

As noted above, superoxide radical rapidly disproportionate to generate hydrogen peroxide. We used ferrous ion oxidation in the presence of xylenol orange to detect hydrogen peroxide in our assays.50 The resulting ferric ion—xylenol complex has an apparent extinction coefficient of 2.24 × 105 M−1 cm−1 at 560 nm. Incubation of 1-HP (100 μM), NADPH (200 μM), and CYPOR (0.05 U/mL) for 4 h at 24 °C followed by addition to a solution containing xylenol orange and ammonium ferrous sulfate gave a strong absorbance at 560 nm. In contrast, no significant absorbance above background was observed in a parallel reaction spiked with the hydrogen peroxide-destroying enzyme catalase. Overall, the evidence indicated that 1-HP mediates the generation of superoxide radical and hydrogen peroxide in conjugation with NADPH alone or the CYPOR enzyme system.

Evidence That 1-HP Mediates Oxidant Generation in Cells. Finally, we examined whether 1-HP mediated the generation of oxidants in cells. In these experiments, RAW264.7 cells were incubated with 1-HP (5–30 μM), and ROS generation was probed by either H2DCFDA (20 μM) or DHE (20 μM). H2DCFDA and DHE are well-known profiluorescent probes for ROS.51 H2DCFDA is converted to the fluorescent dye dichlorofluorescein (DCF) by various oxidants including HO•, CO2•+, NO2•, cytochrome c, low molecular weight organic radicals, and H2O2 in the presence of various catalysts such as heme iron.51–54 DHE displays distinct reactivity and is converted to the fluorescent products 2-hydroxyethidium (HE) and ethidium (E+) by O2•− and HO•, ONOO−, or H2O2, respectively.51,53,54 Fluorescent microscopy experiments cannot distinguish HE and E+.51,54 Confocal microscopy revealed concentration-dependent increases in the fluorescence of DCF or HE in cells treated with 1-HP, consistent with intracellular generation of ROS by 1-HP (Figure S). It is known that H2DCFDA alone can induce green fluorescence via autoxidation and peroxidatic mechanisms.51,54 This type of chemistry may contribute to the background fluorescence observed in the control samples containing no 1-HP. However, it is important to emphasize that we observed increases in DCF fluorescence that are above background levels and are dependent upon the presence and concentration of 1-HP (Figure S). The fluorescence increases of both DCF and HE generated by 1-HP (30 μM) were comparable to those generated by the redox-cycling quinone DMNQ (30 μM, Figure SD). It is important to acknowledge that serious problems such as the potential for redox cycling by these probes preclude quantitative conclusions. Furthermore, the nonselective reactivity of these probes does not allow implication of a specific oxidant in the fluorescence increases induced by 1-HP in cells. Nonetheless, the results provided preliminary evidence that 1-HP mediates the generation of intracellular oxidants. The results of these cellular studies forge a tentative link to our own in vitro studies described above and are in line with earlier studies that providing evidence that 1-HP mediates the generation of H2O2 in tracheal epithelial cells.55 and ESR data showing that phenazine 1-carboxylic acid leads to production of O2•− in AS49 cells.58

CONCLUSIONS

We have presented evidence that 1-HP mediates the net transfer of electrons from NADPH to molecular oxygen. This process can be facilitated by the enzyme CYPOR. The resulting generation of ROS may contribute to the properties of 1-HP as a virulence factor for P. aeruginosa. Our results are consistent with previous studies that measured the ability of 1-HP to induce ciliary dysfunction in mammalian tracheal epithelial cells.55 The authors of this earlier work concluded that 1-HP acted by a mechanism involving the production of H2O2, although this was ascribed to an ability of the compound to activate a respiratory burst in neutrophils rather to direct redox cycling.56 Our results suggesting that 1-HP is susceptible to enzymatic reduction by CYPOR are generally in line with a recent study showing that 1-HP, pyocyanin, and phenazine 1-carboxylic acid serve as electron shuttles that promote anaerobic survival of P. aeruginosa.57 It was suggested that phenazine 1-carboxylic acid serves in this capacity by accepting reducing equivalents from some as yet unidentified enzyme in P. aeruginosa.58 Finally, it is interesting to note that generation of ROS by the virulence factor phenazine 1-carboxylic acid has been detected in cellular assays,57 although it has been reported that this agent is incapable of NAD(P)H-driven redox cycling.58 Redox-driven generation of ROS by small organic molecules is important in drug action, toxicology, and high-throughput screening assays.59–61 There is a growing awareness that chemical structures beyond classical motifs such as quinones and paraquat can undergo redox cycling.21,60–62 The results reported here contribute to a broader understanding of the structural motifs that are capable of redox-driven ROS generation under physiological conditions.

ASSOCIATED CONTENT

Supporting Information

Figure S1: Control reactions for DNA strand cleavage by 1-hydroxyphenazine. Figure S2: Cytochrome c assay for the detection and quantitation of superoxide radical. Figure S3: Ferrous ion oxidation (FOX) assay for the detection and quantitation of hydrogen peroxide. Figure S4: 1H and 13C NMR spectra of 1-hydroxyphenazine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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**ABBREVIATIONS**

- CYPOR, NADPH:cytochrome P450 reductase; NBT, nitroblue tetrazolium chloride; SOD, superoxide dismutase; CAT, catalase; Des, desferal; 1-HP, 1-hydroxyphenazine; DHE, dihydroethidium; HE, 2-hydroxyethidium; H2DCFDA, dichlorodihydrofluorescein diacetate; DCF, dichlorofluorescein; HE, 2-hydroxyethidium; ROS, reactive oxygen species

**REFERENCES**


(37) Kim, W., and Gates, K. S. (1997) Evidence for thiol-dependent production of oxygen radicals by 4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione (oltipraz) and 3H-1,2-dithiole-3-thione: possible relevance to the anticarcinogenic properties of 1,2-dithiole-3-thiones. *Chem. Res. Toxicol.* 10, 296–301.


