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Binding of myeloperoxidase to bacteria: effect on hydroxyl radical formation and susceptibility to oxidant-mediated killing

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Abstract

Neutrophils form superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) and release myeloperoxidase (MPO) during ingestion of microbial pathogens. MPO, which adheres to some bacteria, catalyzes the formation of HOCl from H_2O_2, thereby enhancing $H_2O_2/O_2^{\cdot-}$ microbicidal activity. Hydroxyl radical ($HO^{\cdot}$), also is an important contributor to H_2O_2 and $O_2^{\cdot-}$ microbicidal activity. MPO decreases iron-catalyzed $HO^{\cdot}$ production but also leads to $HO^{\cdot}$ production through the reaction of $O_2^{\cdot-}$ and HOCl. We hypothesized that binding of MPO to bacteria could alter the magnitude and site of $HO^{\cdot}$ production upon organism exposure to $O_2^{\cdot-}/H_2O_2$. Incubation of MPO with *Escherichia coli* and *Pseudomonas aeruginosa* resulted in stable association of MPO with the bacteria which enhanced their susceptibility to killing by $O_2^{\cdot-}/H_2O_2$. In the absence of MPO preincubation exposure of *E. coli*, but not *P. aeruginosa* to $O_2^{\cdot-}/H_2O_2$, led to iron-catalyzed $HO^{\cdot}$ generation. This was associated with different amounts of redox active iron in the two types of bacteria. MPO preincubation slightly decreased $HO^{\cdot}$ detected with *E. coli*, but markedly increased $HO^{\cdot}$ formation with *P. aeruginosa*. This likely resulted from decreased iron-catalyzed $HO^{\cdot}$ production counterbalanced by increased iron-independent $HO^{\cdot}$ formation. MPO preincubation did not effect bacterial killing by a system which generated only H_2O_2, precluding MPO-dependent $HO^{\cdot}$ formation. These data are consistent with a possible role for MPO-derived $HO^{\cdot}$ in the augmentation of bacterial killing by this enzyme.

Keywords: Myeloperoxidase; Hydroxyl radical; Superoxide; Hypochlorous acid; (*E. coli*); (*P. aeruginosa*)

1. Introduction

Among the mechanisms used by neutrophils to kill ingested microorganisms is the generation of microbicidal oxidant species [1]. Phagocytosis induces the assembly and activation of a membrane-associated NADPH-dependent oxidase complex. This complex univalently reduces O_2 to superoxide anion ($O_2^{\cdot-}$) [1]. At physiologic pH, $O_2^{\cdot-}$ rapidly dismutes to form hydrogen peroxide (H_2O_2) [2].

Neutrophil $O_2^{\cdot-}/H_2O_2$ formation is accompanied by the coordinate release of a variety of cytosolic granule components into the developing phagosome [3]. Among these is myeloperoxidase (MPO) [1]. MPO is a heme-containing enzyme that catalyzes the formation of hypochlorous acid (HOCl) from H_2O_2 and Cl^- [2]. HOCl is a potent oxidant that is cytotoxic for many microbial species [2]. MPO, presumably via its ability to generate HOCl, has been repeatedly shown to enhance the ability of $O_2^{\cdot-}/H_2O_2$-generating systems to kill a variety of eukaryotic and prokaryotic cells [1,2].

In addition to a role for $O_2^{\cdot-}$, H_2O_2, and HOCl, the formation of hydroxyl radical ($HO^{\cdot}$) has also been implicated as an important contributor to neutrophil-mediated cytotoxicity [4]. Hydroxyl radical is an extremely reactive oxidant with the potential to damage nearly all types of biomolecules (proteins, DNA, lipids and carbohydrates). Its rate of reaction with most substrates is diffusion limited ($\approx 10^9\ M^{-1}\ s^{-1}$). This high reactivity usually necessitates that $HO^{\cdot}$ be generated in close proximity to target molecules for it to cause injury. However, it may cause cell injury at sites distant to its formation via the initiation of a free radical reaction cascade [5].

Most studies exploring the role of $HO^{\cdot}$ in neutrophil-mediated cytotoxicity have focused on its formation via

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the iron-catalyzed reaction of $O_2^{\cdot-}$ with H_2O_2: the Haber–Weiss reaction [4].

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$\underline{H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^- + Fe^{3+}}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow HO^{\cdot} + OH^- + O_2$$

Neutrophils lack an iron catalyst for such a reaction [6,7]. In circumstances in which iron-catalyzed $HO^{\cdot}$ formation contributes to target cell injury it appears that it is the target cell itself which contributes the required iron catalyst [8,9]. The result is intracellular $HO^{\cdot}$ formation. Studies from our laboratories [10] as well as that of Winterbourn [11] indicate that release of MPO may inhibit neutrophil associated Haber–Weiss-mediated $HO^{\cdot}$ formation by decreasing the availability of H_2O_2 for this reaction.

In contrast to its impact on the Haber–Weiss reaction, we recently showed that phagocyte MPO release can also promote $HO^{\cdot}$ generation through the reaction of MPO-derived HOCl with $O_2^{\cdot-}$ [7].

$$HOCl + O_2^{\cdot-} \rightarrow HO^{\cdot} + O_2 + Cl^-$$

In order for the $HO^{\cdot}$ formed through this process to have any significant role in neutrophil microbicidal activity, it would need to be generated in close proximity to the bacterial surface. MPO is a highly cationic protein, which will rapidly adhere to negatively charged bacterial or fungal surfaces [12,13]. Electron microscopic studies of bacteria contained within neutrophil phagosomes reveal intense deposition of MPO on the bacterial surface [14]. Several laboratories have demonstrated that conditions which lead to coating of bacterial surfaces with MPO or other peroxidases augment killing of these organisms by $O_2^{\cdot-}/H_2O_2$ [12,15–19], as well as other phagocyte cells which lack MPO [16,18]. The general assumption has been that this process is due to the direct effects of HOCl on the microbe.

Based on the above, we hypothesized that the binding of MPO to microbial surfaces could have multiple effects on the magnitude and site of $HO^{\cdot}$ formation associated with neutrophil ingestion of microbial pathogens. It could decrease intracellular iron-catalyzed $HO^{\cdot}$ formation while promoting iron-independent $HO^{\cdot}$ production at the microbial surface. Here we use two bacterial pathogens, *Escherichia coli* and *Pseudomonas aeruginosa*, to examine this hypothesis using cell-free enzymatic oxidant-generating systems. The potential impact of these reactions on microbial killing is also explored.

2. Materials and methods

2.1. Bacteria

The following strains of *Escherichia coli* were utilized: HB101; 055:B5 (CAS strain); Cook strain (ATCC 8739); 0127:B8; ATCC 23589; ATCC 11775; and ATCC 23738. *Pseudomonas aeruginosa* strains used were: PA01; PA103; PA29859; and PA 21746. Clinical isolates of *Staphylococcus aureus* were also examined. All bacterial strains were kindly provided by Dr. Charles Cox, Department of Microbiology, University of Iowa College of Medicine, Iowa City, IA. Each isolate was subcultured daily on tryptic soy agar plates (Difco, Laboratories, Detroit, MI) at 37°C. On the day of experimentation, the organism of interest was transferred to tryptic soy broth (TSB) and placed at 37°C in a Shaker Incubator (model G-24, New Brunswick Scientific, Edison, NJ) until the culture reached mid log phase (2–4 h), which was monitored by measuring the A_{600} of the bacterial suspension. Organisms were then pelleted ($1850 \times g$, 10 min) and repetitively washed in Hanks' Balanced Salt Solution without phenol red, but containing 1.3 mM Ca^{+2}, and 0.5 mM Mg^{+2} (HBSS, University of Iowa Cancer Center, Iowa City, IA). The bacteria were then suspended at the desired concentration, based on correlations between A_{600} and colony forming units (cfu) previously determined by serial dilution and plating on tryptic soy agar, and then placed at 4°C until use. For experiments in which organisms were lysed prior to study, the bacterial cell suspension was subjected to sonication $\times 3$ for 30 seconds on ice using an ultrasonic disrupter (Microson XL, Heat Systems, Farmington, NY) at a setting of 11–12. The sample was then frozen to −80°C, thawed, and centrifuged ($1750 \times g$, 10 min) to clarify the sample. The supernatant was then removed and used for lysate studies. In the case of assessment of antioxidant enzyme activity, bacteria were suspended in 50 mM potassium phosphate buffer prior to sonication.

2.2. Bacterial incubation with myeloperoxidase

Purified human MPO was kindly provided by Dr. William Nauseef (Department of Internal Medicine, University of Iowa College of Medicine and Research Service, VA Medical Center, Iowa City, IA). Organisms that had been grown to log phase were prepared as above and then suspended in HBSS at a concentration of 5×10^{10} cfu/ml in the presence of 200–1000 *o*-dianisidine units/ml [20] of human MPO for 1 h at 37°C. The organisms were washed $\times 3$ in HBSS and then were resuspended to the desired concentration in HBSS. MPO activity associated with each organism or with the stock enzyme preparation was quantitated on the basis of the ability of the enzyme to catalyze the oxidation of *o*-dianisidine as previously described [20]. For quantitating organism associated MPO, $(1–2) \times 10^9$ cfu were added to the *o*-dianisidine assay. Higher concentrations resulted in turbidity which precluded accurate determination of MPO activity. Bacteria-associated MPO activity was calculated based on rates of *o*-dianisidine oxidation observed that day using a known amount of purified MPO. In the absence of preincubation

with MPO, bacterial samples failed to oxidize *o*-dianisidine. The presence of bacteria did not alter the ability of the assay to detect known amounts of MPO activity.

2.3. Spin trapping

Organisms were suspended (5×10^9/ml) in HBSS containing 0.64 mM lumazine (Sigma Chemical, St. Louis, MO), 10 mM α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (4-POBN, Sigma), 140 mM ethanol (Midwest Grain Products, Pekin, IL), and 10 mM diethylenetriaminepentaacetic acid (DTPA, Aldrich, Milwaukee, WI). The HBSS had been pretreated with chelating resin (Sigma) to decrease the presence of adventitious iron [21]. Following the addition of bovine milk xanthine oxidase (final concentration 4 milliunits/ml, Boehringer Mannheim, Indianapolis, IN). The suspension was transferred to an EPR flat cell and placed into the cavity of the EPR spectrometer (Bruker Model ESP 300, Bruker, Karlsruhe, Germany). EPR spectra were then serially monitored at 25°C. Spectrometer settings were: microwave power 20 mW, modulation frequency 100 kiloHertz (kHz), modulation amplitude 0.94 Gauss (G), time constant 0.164 s, and the gain 5.0×10^5. Detection of 4-POBN/$^{\cdot}CHOHCH_3$ ($a^N = 15.5$ G, $a^H = 2.6$ G) was equated to the formation of $HO^{\cdot}$ [7]. The xanthine oxidase had also been pretreated with chelating resin as previously described to remove adventitious iron [22].

2.4. Bacterial killing assays

Organisms were suspended at a concentration of 10^4 cfu/ml in HBSS (*E. coli*) or a mixture of 40% (v/v) HBSS and 60% (v/v) TSB (*P. aeruginosa*) to which was added 1.6 mM lumazine or 5.5 mM glucose. Xanthine oxidase (lumazine containing suspensions) or glucose oxidase (glucose supplemented suspensions) were then added and the suspensions were incubated at 37°C. Aliquots were removed and serially diluted in HBSS (*E. coli*) or HBSS/TSB (*P. aeruginosa*) at 0, 30 and 60 min of incubation and then inoculated onto tryptic soy agar plates that were in turn placed at 37°C. Resultant colonies were then enumerated following overnight incubation. In each experiment, parallel incubations were performed in which a suspension of organisms were incubated in the absence of exogenous xanthine oxidase or glucose oxidase. The experiments with *P. aeruginosa* were performed in the mixture of HBSS and TSB because it was found that suspension of *P. aeruginosa* in HBSS alone resulted in an unsatisfactory decrease in cfu of the control (non-xanthine-oxidase or glucose-oxidase treated) organisms over the time-course of the experiment.

2.5. Antioxidant enzyme activity gels

Staining of native gels for catalase and superoxide dismutase activity were performed according to previously described methods [23,24]. Briefly, lysates from 1×10^9 bacteria were suspended in 50 mM potassium phosphate buffer, were loaded onto 10% (SOD) or 8% (catalase) native nondenaturing polyacrylamide gels, and then subjected to electrophoresis [23,24]. Gels were then stained for SOD or catalase enzymatic activity. SOD activity was assessed by incubating the gels in 2.45 mM Nitro blue tetrazolium (NBT) in distilled water for 20 min following which they were washed and placed in the presence of 28 μM riboflavin and 28 mM tetramethylethlenediamine. After 15 min the gels were washed and exposed to intense visible light, which results in the generation of superoxide. SOD activity results in the formation of clear areas in the gel in the setting of a purple background due to the superoxide mediated reduction of NBT [24]. Catalase activity was assessed incubating the gel in 0.003% H_2O_2 (10 min). After washing in H_2O, the gels were exposed to a solution containing 2% $FeCl_3$ and 2% ferricyanide ($K_3Fe(CN)_5$). Catalase activity on the gel is visualized as clear areas on a green background [23]. In each case, gels contained positive controls composed of human MnSOD (provided by Dr. Larry Oberley, Radiation Research Laboratories, University of Iowa, Iowa City, IA), human CuZn-SOD (Sigma) or bovine liver catalase (Calbiochem, San Diego, CA).

2.6. Redox active iron

For purposes of this assay, redox active iron is defined as the ability to generate ascorbate radical, as detected by EPR spectroscopy, using a modification of previous work in which this technique was used to detect catalytic iron in biologic systems [21,25,26]. Ascorbic acid (125–250 μM) was added to bacterial lysates (10^9/ml) in phosphate buffer (pH 7.0) from which adventitious metals had been previously removed by batch treatment with chelating resin [21]. The samples were then immediately transferred into an EPR flat cell, placed into the EPR cavity, and the concentration of ascorbate radical quantitated [26]. In the presence of up to 10^9/ml *P. aeruginosa* the ascorbate radical signal was found to be stable for up to 15 min. In contrast, with *E. coli*, concentrations of the organism above 10^9/ml resulted in a rapid loss of EPR-detectable ascorbate radical generated by the reaction of H_2O_2 and $FeSO_4$ in the presence of ascorbate. Therefore all experiments reported were performed at concentrations of both types of organisms below this critical concentration.

3. Results

3.1. Binding of myeloperoxidase to bacteria

Escherichia coli, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are important human pathogens and neutrophils appear to play an important role in protecting

the host from them. Therefore, the ability of purified human MPO to bind to their surface was examined. Organisms that had been grown to log phase were washed and suspended (5×10^{10}/ml) in HBSS alone or in HBSS containing 200–1000 *o*-dianiside units of human MPO/ml. These bacterial suspensions were incubated at 37°C for 1 h following which they were repetitively washed and bacteria-associated MPO activity determined. MPO associated with the bacteria was detected with all seven strains of *E. coli* and four strains of *P. aeruginosa* examined. MPO activity ranged from 60–204 *o*-dianisidine units/10^{10} organisms (median = 100 units/10^{10} organisms, $n = 7$) for the *E. coli* and was 6.7–53 *o*-dianisidine units/10^{10} organisms for the *P. aeruginosa* strains (median = 13.2, $n = 7$). In contrast, no MPO association with *S. aureus* could be demonstrated. Western blotting using antibody to MPO was consistent with the results of the MPO activity assays (data not shown). Accordingly, no additional experiments with *S. aureus* were performed.

Table 1
Effect of bacterial coating with MPO on susceptibility to killing by oxidant generating systems

	E. coli		*P. aeruginosa*	
	XO/L (17)	G/GO (11)	XO/L (9)	G/GO (13)
−MPO coating	54±4	58±10	76±5	37±10
+MPO coating	38±4 *	62±12	47±8 * *	43±12

Shown are percentage of cfu (mean ± S.E.M.) relative to untreated control of *E. coli* (strain HB101) or *P. aeruginosa* (strain PA01) following a 60 min exposure to the reaction of xanthine oxidase and lumazine (XO/L) or glucose and glucose oxidase (G/GO) as a function of whether the organism had or had not been previously 'coated' with human MPO. Numbers in parenthesis are the number of experiments performed with that oxidant generating system. All percentages are significantly less than the untreated control ($P < 0.05$).
* significantly decreased viability compared to non-MPO coated organism ($P < 0.0005$).
* * significantly decreased viability compared to non-MPO coated organism ($P < 0.05$).

3.2. MPO binding enhances killing of E. coli and P. aeruginosa by $O_2^{\cdot-}/H_2O_2$

Having demonstrated the ability of MPO to associate with both *E. coli* and *P. aeruginosa*, the possibility that this process would enhance the susceptibility of these organisms to killing by $O_2^{\cdot-}/H_2O_2$ was examined. Aerobic oxidation of xanthine, hypoxanthine, or lumazine by xanthine oxidase results in the reduction of ambient O_2 to both $O_2^{\cdot-}$ and H_2O_2. H_2O_2 is formed both as a result of: (i) the univalent reduction of O_2 to $O_2^{\cdot-}$ with subsequent dismutation of $O_2^{\cdot-}$ to H_2O_2; and (ii) via the direct divalent reduction of O_2 [27,28]. The reaction of xanthine oxidase with the above substrates is a common cell-free model used in assessing bacterial susceptibility to killing by phagocyte-derived oxidants [29]. Compared to other xanthine oxidase substrates, oxidation of lumazine by xanthine oxidase leads to a greater percentage of O_2 reduction occurring univalently rather than divalently [28]. Since almost all of the H_2O_2 generated by human neutrophils occurs as the result of initial $O_2^{\cdot-}$ formation [30,31], oxidation of lumazine by xanthine oxidase would appear to most closely resemble oxidant generation occurring within the neutrophil phagosome. Therefore, the reaction of xanthine oxidase and lumazine was employed in our studies.

In order to be able to assess the impact of MPO coating on bacterial susceptibility to $H_2O_2/O_2^{\cdot-}$ killing concentrations of xanthine oxidase and lumazine which resulted in submaximal killing of *E. coli* and *P. aeruginosa* were ascertained. Previously, we found that inclusion of SOD and catalase in such a reaction mixture prevents bacterial killing, confirming that it occurs via an oxidant-dependent mechanism. Once the desired xanthine oxidase/lumazine concentrations were defined, the susceptibility of organisms 'coated' with MPO relative to control to killing by this system was determined. Both *E. coli* strain HB101, and *P. aeruginosa* strain PA01, which had been preincubated with MPO, demonstrated increased susceptibility to killing (Table 1). Of note, the amount of MPO present in the reaction mixture as contributed by the MPO adherent to the organisms is far less than that routinely employed by others to augment H_2O_2-mediated killing of bacteria [32].

3.3. Detection of hydroxyl radical formation during exposure of bacteria to $O_2^{\cdot-}/H_2O_2$

We previously demonstrated [7] that MPO-derived HOCl will react with $O_2^{\cdot-}$ to generate $HO^{\cdot}$, raising the possibility that the generation of this free radical could contribute to the MPO-mediated enhancement of bacterial killing reflected in Table 1. As a first step in testing this hypothesis, we examined the extent to which $HO^{\cdot}$ was formed when control and MPO-coated organisms were exposed to the xanthine oxidase/lumazine $O_2^{\cdot-}/H_2O_2$-generating system. Hydroxyl radical formation was assessed using a spin trapping system composed of the combination of ethanol and the spin trap 4-POBN. Previous work has shown that detection of the α-hydroxyethyl radical spin adduct of 4-POBN is a sensitive and highly specific indicator of $HO^{\cdot}$ production [7,33].

As shown in Fig. 1 (trace A), EPR spectra obtained during the exposure of control (not MPO-incubated) *E. coli* to xanthine oxidase and lumazine in the presence of 4-POBN and ethanol, consistently yielded evidence of the spin adduct (4-POBN/$^{\cdot}CH(OH)CH_3$) indicative of the formation of $HO^{\cdot}$. This was true for each of the seven *E. coli* strains examined (data not shown). Organisms pretreated with deferoxamine (1 mM) for 1 hour and then washed free of the compound demonstrated decreased formation of $HO^{\cdot}$ upon subsequent exposure to xanthine oxidase/lumazine (Fig. 1, trace C). This suggests $HO^{\cdot}$ was generated via an iron-dependent reaction process such

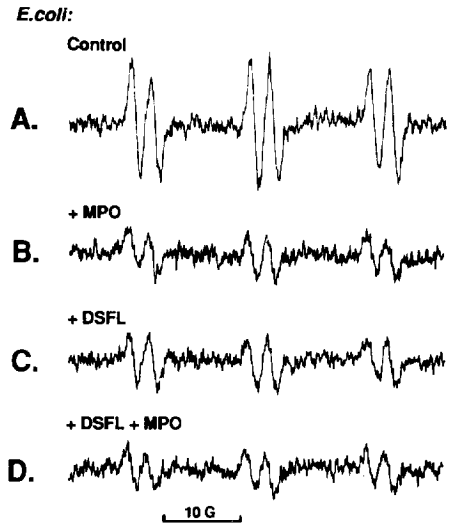

Fig. 1. Shown are EPR spectra representative of 5–9 separate experiments in which *E. coli* strain HB101, which had not been preincubated with MPO (trace A) or which had been preincubated with MPO (trace B,) were exposed to the xanthine oxidase/lumazine system in the presence of 4-POBN/ethanol. Also shown are the effects of preincubation of both group of organisms with 1 mM deferoxamine prior to their exposure to xanthine oxidase/lumazine (traces B and D). EPR spectra are those of 4-POBN/ $^{\cdot}CH(OH)CH_3$. Results were similar for other strains of *E. coli* and *P. aeruginosa* tested.

as the Haber–Weiss reaction. Experiments in which similar concentrations of *P. aeruginosa* strains were employed usually yielded EPR spectra of 4-POBN/ $^{\cdot}CH(OH)CH_3$ which were barely above background noise indicating the generation of minimal amounts of $HO^{\cdot}$ (Fig. 2, trace B). Reproducibly, the magnitude of $HO^{\cdot}$ detected was less than observed with *E. coli* (Fig. 2, trace A).

The differences between the magnitude of $HO^{\cdot}$ generated upon exposure of the two different bacterial species to the $O_2^{\cdot-}/H_2O_2$-generating system could not be explained on the basis of variations in the stability of 4-POBN/ $^{\cdot}CH(OH)CH_3$ in the presence of *P. aeruginosa*

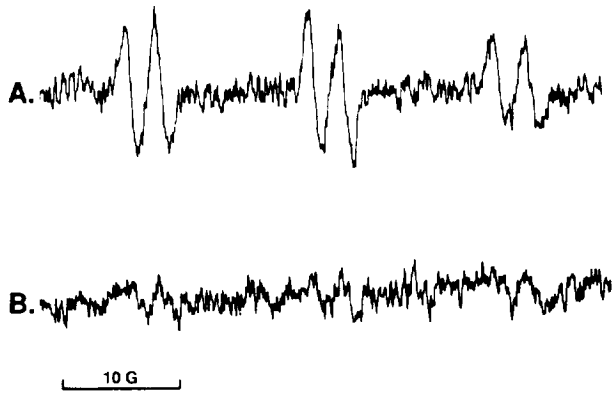

Fig. 2. EPR spectra representative of 8 separate experiments in which *E. coli* (strain HB101) or *P. aeruginosa* (strain PA01) were exposed to xanthine oxidase/lumazine in the presence of 4-POBN/ethanol. Results were similar for other strains of *E. coli* and *P. aeruginosa* tested.

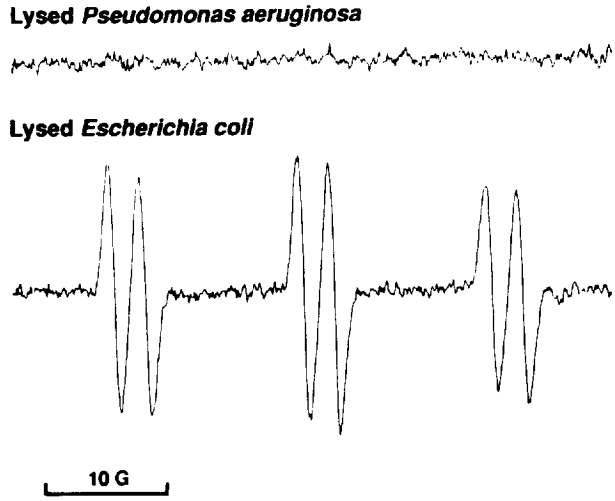

Fig. 3. EPR spectra representative of 3 experiments in which lysates of *E. coli* strain HB101 or *P. aeruginosa* strain PA01 were exposed to xanthine oxidase/lumazine in the presence of 4-POBN/ethanol.

compared to *E. coli*. Addition of either organism to a solution of 4-POBN/ $^{\cdot}CH(OH)CH_3$, generated previously by reacting H_2O_2 with Fe^{2+} in the presence of 4-POBN and ethanol, failed to demonstrate any difference in the stability of the spin adduct when its EPR spectrum was monitored over time (data not shown).

The different results obtained with *E. coli* and *P. aeruginosa* could also not be explained on the basis of differences in the permeability of the organisms' membranes to the spin trapping compounds. Addition of xanthine oxidase/lumazine to bacterial lysates derived from the two organisms in the presence of 4-POBN and ethanol again yielded either no (Fig. 3) or at best much lesser amounts (data not shown) of 4-POBN/ $^{\cdot}CH(OH)CH_3$ with *P. aeruginosa* relative to *E. coli* (Fig. 3).

These data suggested that there was a true difference between the two bacterial species in the amount of $HO^{\cdot}$ formed upon exposure to a source of $O_2^{\cdot-}/H_2O_2$. Hassett and colleagues recently reported that *P. aeruginosa* possesses greater levels of important antioxidant enzymes such as catalase and SOD compared to *E. coli* [34]. Since such a situation could contribute to differences in $HO^{\cdot}$ formation via the Haber–Weiss reaction, we determined the activity of catalase and SOD in the two bacterial species when they were grown under our experimental conditions. In contrast to the results of Hassett et al [34], no differences in SOD or catalase activity were detectable when assessed by 'activity gels' (Fig. 4).

A difference in their content of catalytic iron was an alternative explanation for the difference in $HO^{\cdot}$ formation observed upon exposure of the two types of organisms to $O_2^{\cdot-}/H_2O_2$. Interaction of ascorbate with redox active iron generates ascorbate radical [25,26]. The intensity of ascorbate radical detected by EPR spectroscopy correlates with the amount of such iron present [26]. Thus, ascorbate was added to lysates from an equivalent number of *E. coli* and

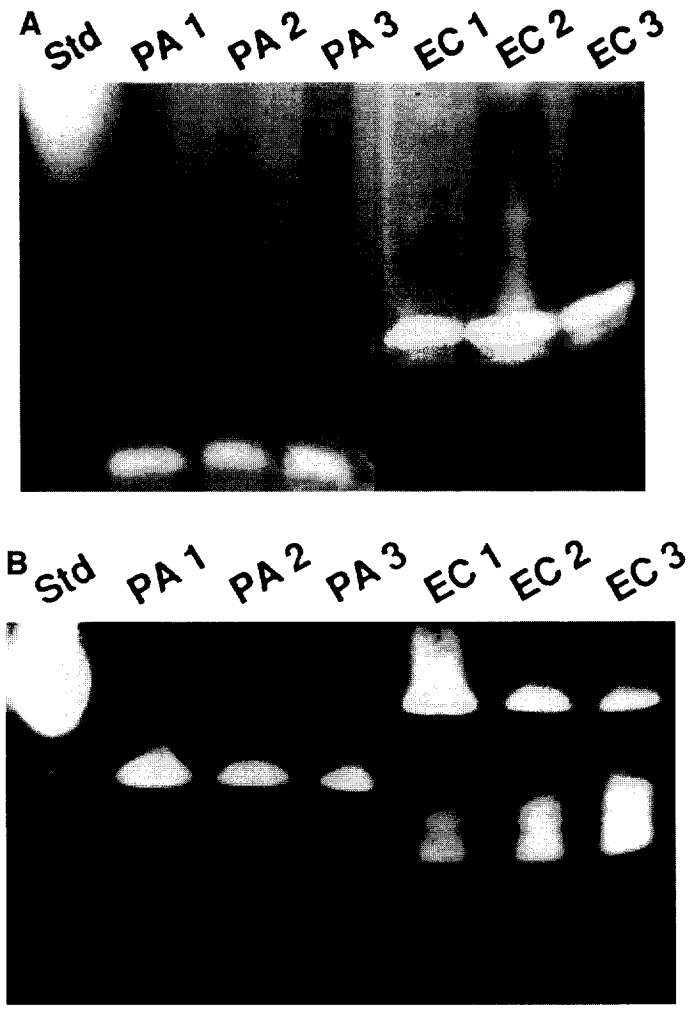

Fig. 4. SOD (A) and catalase (B) activity gels demonstrating equal activity of both enzyme classes among three separate strains of *E. coli* (HB101, 055:B5, and 0127:B8) and *P. aeruginosa* (PA01, PA 103, PA21746, respectively labeled PA1, PA2, PA3). Bovine catalase or human MnSOD are included as positive controls.

P. aeruginosa following which ascorbate radical was quantitated by EPR. Results using *P. aeruginosa* lysates (Fig. 5, trace B) were not generally above that observed with the buffer alone (Fig. 5, trace A). The *E. coli* lysates consistently yielded greater amounts of ascorbate radical suggesting a greater presence of redox active iron (Fig. 5, trace C). In order to eliminate the possibility that these differences resulted from decreased stability of the ascorbate radical in the *P. aeruginosa*-derived lysate rather than a difference in iron, known amounts of iron were added to lysate samples from the two organisms following which ascorbate was added. Under the above conditions there was no difference in the magnitude of the resulting ascorbate radical (data not shown). However, *E. coli* concentrations above 10^9 organisms/ml resulted in a rapid decrease in the half-life of the ascorbate radical (data not shown). In contrast, the radical was quite stable in the presence of lysate derived from up to 10^{10}/ml of *P. aeruginosa*. Thus, if anything the amount of redox active iron in the *E. coli* compared to *P. aeruginosa* lysates may be underestimated by this method.

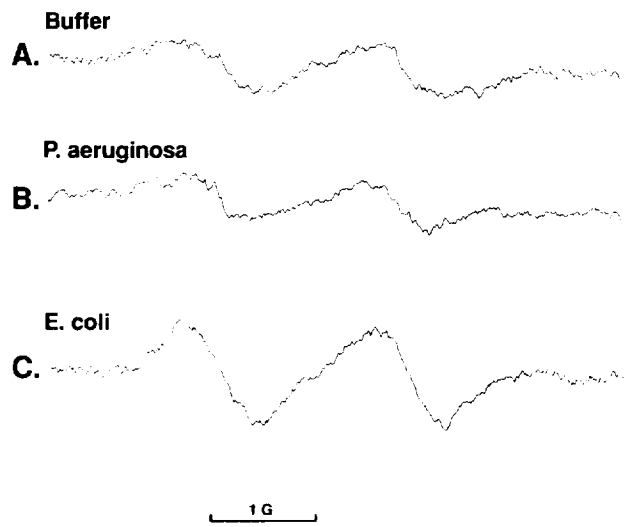

Fig. 5. Shown are EPR spectra indicative of ascorbate radical (a^H = 1.8 G) which were obtained upon the addition of ascorbic acid to buffer (top spectrum) or lysates derived from *P. aeruginosa* strain PA01 (middle spectrum) or *E. coli* strain HB101 (bottom spectrum). Results are representative of 3 separate experiments.

3.4. Effect of MPO binding to bacteria on hydroxyl radical generation

As noted earlier, MPO can promote the formation of $HO^{\cdot}$ via an iron-independent reaction [7] but inhibit $HO^{\cdot}$ generation via the iron-catalyzed Haber–Weiss reaction [10,11]. Therefore, we examined the effect of the binding of MPO to *E. coli* and *P. aeruginosa* on the magnitude of $HO^{\cdot}$ formed upon exposure of the organism to $O_2^{\cdot-}/H_2O_2$. Bacteria were grown to log phase and then incubated with MPO under the conditions which earlier had resulted in binding of MPO. The bacteria were then harvested, exposed to xanthine oxidase and lumazine in the presence of 4-POBN and ethanol; the resulting 4-POBN/$^{\cdot}CH(OH)CH_3$ was quantitated by EPR. The magnitude of $HO^{\cdot}$ formed with these MPO-coated organisms was then compared to results obtained with the same organisms prepared under the same conditions except that they were preincubated in buffer that did not contain MPO. As reflected in Fig. 1 (trace B vs. trace A), MPO coating of *E. coli* generally produced a decrease in the quantity of $HO^{\cdot}$ that was spin trapped. These results could not be explained on the basis of MPO-mediated alterations in the stability of the 4-POBN/$^{\cdot}CH(OH)CH_3$ spin adduct. We previously showed that this spin adduct is stable in the presence of MPO-derived oxidants at concentrations greater than generated under these experimental conditions [35]. In contrast to results with non-MPO-coated organisms (Fig. 1, traces A and C), pretreatment of MPO-exposed *E. coli* with deferoxamine had no major effect on the amount of $HO^{\cdot}$ formed (Fig. 1, traces B and D).

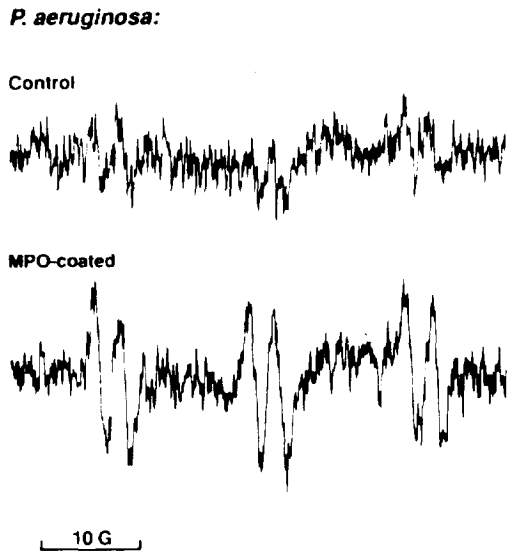

Fig. 6. EPR spectra representative of 8 experiments in which *P. aeruginosa* strain PA01 which had not (top spectrum) or had (bottom spectrum) been incubated with MPO (coated) were exposed to the xanthine oxidase/lumazine system in the presence of 4-POBN/ethanol. The spectrum observed in the bottom trace is that of 4-POBN/$^{\cdot}CH(OH)CH_3$. Similar results were obtained with all *P. aeruginosa* strains examined.

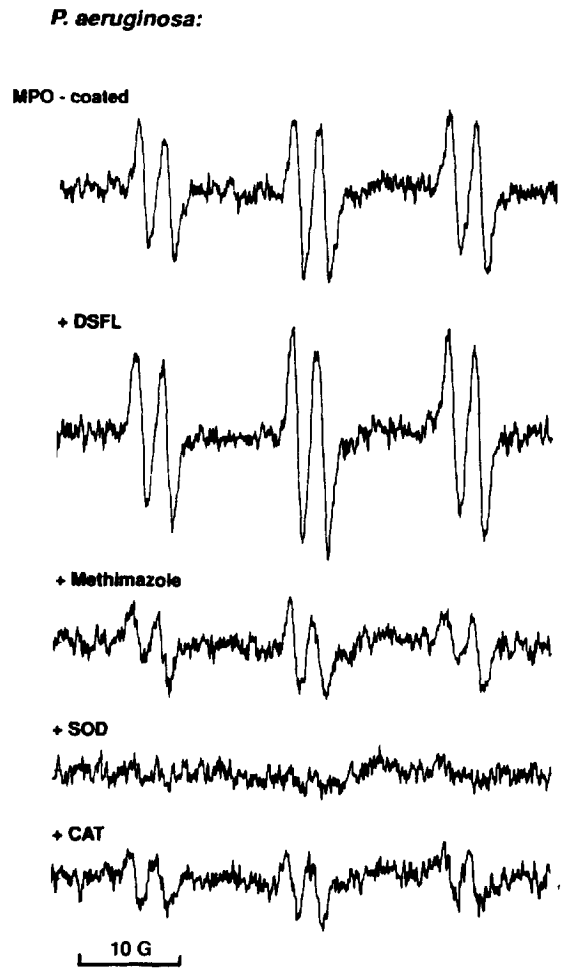

Fig. 7. Shown are the effects of preincubation of MPO-coated *P. aeruginosa* (strain PA01) with deferoxamine (DSFL), or the co-incubation with methimazole (1 mM), SOD, or catalase on the magnitude of $HO^{\cdot}$ spin trapped upon the addition of xanthine oxidase and lumazine to the bacterial suspension in the presence of 4-POBN/ethanol. Results are reflective of 4 experiments.

When the above experiments were repeated using MPO-coated *P. aeruginosa* different results were obtained. MPO-coated organisms generated greater amounts of $HO^{\cdot}$ upon exposure to the xanthine oxidase/lumazine system than control organisms (Fig. 6). The magnitude of $HO^{\cdot}$-derived spin adducts was inhibited when the MPO-coated organisms were concurrently incubated with catalase, SOD, or methimazole, but not deferoxamine (Fig. 7). These data are consistent with $HO^{\cdot}$ generation via the MPO-dependent mechanism rather than the Haber–Weiss reaction.

3.5. Could $HO^{\cdot}$ formation contribute to the enhanced susceptibility of MPO-coated bacteria to $O_2^{\cdot-}/H_2O_2$-dependent killing?

We next sought to examine whether the MPO-dependent formation of $HO^{\cdot}$ we detected could contribute to

enhanced killing of MPO coated organisms or whether this was solely the result of direct HOCl-mediated injury. Although studying the effects of oxidant scavengers has traditionally been utilized to identify the biologic role of different oxidant species, this approach was not employed. Since MPO-mediated $HO^{\cdot}$ generation is dependent on HOCl, 'HOCl scavengers' would be expected to inhibit MPO enhanced killing regardless of whether it was mediated by HOCl or $HO^{\cdot}$ derived from HOCl. Studying the effect of '$HO^{\cdot}$ scavengers' also seemed suboptimal given the lack of specificity of oxidant scavengers as a whole, as well as uncertainty as to their ability to reach the potential sites of $HO^{\cdot}$ formation occurring with viable bacteria [36,37].

Instead, we took advantage of the fact that MPO-dependent formation of $HO^{\cdot}$ only occurs if there is the coexistent presence of $O_2^{\cdot-}$. [7,38]. On the other hand MPO will generate HOCl from H_2O_2 in the absence of $O_2^{\cdot-}$. Therefore, we hypothesized that if the formation of $HO^{\cdot}$ was involved in the augmented killing of MPO-coated bacteria, this enhancement should be decreased when a system that generates H_2O_2 in the absence of $O_2^{\cdot-}$ (the reaction of glucose/glucose oxidase) is substituted for xanthine oxidase/lumazine. This was in fact observed. In contrast to results obtained with the xanthine oxidase/lumazine system, we were unable to detect any difference in killing of either *E. coli* or *P. aeruginosa* that had been preincubated with MPO relative to non-MPO treated control organisms upon exposure to the glucose/glucose oxidase system (Table 1). This was the case regardless of whether the glucose/glucose oxidase concentration was adjusted to produce modest baseline killing (Table 1), no killing (data not shown), or maximal killing (data not shown). The fact that $HO^{\cdot}$ was not generated by the addition of MPO to this H_2O_2-generating system was confirmed by spin trapping (data not shown).

4. Discussion

MPO has been previously shown to enhance the in vitro cytotoxicity of $O_2^{\cdot-}$ and H_2O_2 [1,2,32,39,40]. The potential of MPO to cause damage to cells appears to be enhanced by its highly cationic nature, resulting in its ability to associate with and coat the negatively charged surface of a variety of target cells [12,13,15]. Such MPO coating of bacteria has been demonstrated with electron microscopy to occur in the neutrophil phagosome [14]. Presumably this process allows an increase in the local concentration of MPO-derived oxidants to which the organism is exposed.

It has generally been assumed that MPO-mediated cytotoxicity results from the ability of MPO to convert H_2O_2 into hypohalous acids, such as HOCl, which then directly mediate the cytotoxicity. We recently demonstrated that the release of MPO during neutrophil production of $O_2^{\cdot-}$ and H_2O_2 with its resultant formation of HOCl also leads to the production of a small amount of $HO^{\cdot}$ through the reaction of $O_2^{\cdot-}$ and HOCl [7]. Although relatively small in quantity, given the reactivity of $HO^{\cdot}$ and the potential for it to be generated in close proximity to a bacterial target, we hypothesized that MPO-dependent $HO^{\cdot}$ production could contribute to MPO microbicidal activity.

Consistent with previous results with other bacterial species [12,13], we found that MPO becomes intimately associated with *E. coli* and *P. aeruginosa* over a 1 h incubation period. This resulted in enhanced susceptibility of both bacterial species to $O_2^{\cdot-}/H_2O_2$-mediated killing. The magnitude of the enhancement is less than reported in studies in which MPO is added along with the enzymatic source of $O_2^{\cdot-}$ and/or H_2O_2. This is not surprising given that the amount of MPO product in our assays is much lower ($\approx$ 1000-fold) than used in other works [32]. The fact that augmented killing was observed attests to the importance of the site at which oxidants are generated to the damage which results. Earlier works of others with MPO [12,13,17] and other peroxidases [18,19] have documented similar effects on other organisms. However, in several cases [12,13] peroxidase binding only decreased the requirement for the peroxidase in the peroxidase/H_2O_2/halide system but did not increase susceptibility to H_2O_2 alone. In contrast, we were unable to detect any bacteria-associated MPO under the same experimental conditions using *S. aureus*. These data are consistent with the importance of the negatively charged bacterial outer membrane, which as a Gram-positive organism *S. aureus* lacks, in MPO association with microorganisms. Ramsey and colleagues have also been unable to detect binding of MPO to *S. aureus* [18].

Using spin trapping we examined the impact of MPO coating of these bacteria on the amount of $HO^{\cdot}$ formed upon their exposure to a flux of $O_2^{\cdot-}$ and H_2O_2. With the *E. coli* strains, deferoxamine-inhibitable $HO^{\cdot}$ formation was detectable in the absence of preincubation with MPO, suggesting an iron-dependent reaction. It has previously been suggested that $HO^{\cdot}$ formation is responsible for the killing of *E. coli* by H_2O_2 [41]. Our data support this hypothesis and we believe that our report is the first direct evidence that $HO^{\cdot}$ is generated upon exposure of *E. coli* to $O_2^{\cdot-}/H_2O_2$.

In spite of the enhanced susceptibility of MPO-coated *E. coli* to $O_2^{\cdot-}/H_2O_2$-mediated killing there was little impact on the magnitude of $HO^{\cdot}$ generated upon their exposure to the xanthine oxidase/lumazine system. This likely resulted from the ability of MPO to inhibit $HO^{\cdot}$ formation occurring via the Haber–Weiss reaction [10,11] counterbalanced by the production of this species through the interaction of MPO-derived HOCl with $O_2^{\cdot-}$ [7,38].

In contrast to the results with *E. coli*, in the absence of MPO preincubation we detected little $HO^{\cdot}$ upon exposure of *P. aeruginosa* to $O_2^{\cdot-}/H_2O_2$. This did not relate to technical difficulties with the spin trapping system but

rather was illustrative of true differences in the amount of $HO^{\cdot}$ formed with the two bacterial species. This could not be explained on the basis of differences in levels of bacterial catalase or SOD activity. In contrast, to the lack of difference in antioxidant enzymes we found that under our growth conditions that *P. aeruginosa* contained considerably lower levels of redox active iron. This could well account for the differences in $HO^{\cdot}$ generation upon exposure of these organisms to $O_2^{\cdot-}/H_2O_2$. Our antioxidant enzyme data are in contrast to a recent report in which *P. aeruginosa* levels of these enzymes, assessed by both the same activity gel-technique employed in this study-and conventional biochemical assays of enzyme activity, were higher than in *E. coli* [34]. Close correlation was observed in this earlier study between the two techniques. Although we did not perform the more quantitative biochemical analysis of SOD and catalase activity it seems unlikely that our results reflect methodologic differences from the earlier work. Since growth conditions influence bacterial antioxidant enzyme expression [34,42–45], it seems likely that the differences in the bacterial growth conditions employed in the two studies are responsible for the different results.

When *P. aeruginosa* were preincubated with MPO, we detected the formation of $HO^{\cdot}$, which appropriately appeared to be the result of a MPO-dependent mechanism; $HO^{\cdot}$ production was inhibited by SOD, catalase, and methimazole, but not deferoxamine. Thus, in the case of *E. coli*, the magnitude of $HO^{\cdot}$ generation detected by spin trapping did not correlate with the enhanced killing of the organism following MPO preincubation whereas with *P. aeruginosa* such an association was observed. One could argue that these data indicate that the formation of $HO^{\cdot}$ plays no role in the ability of MPO 'coating' to enhance $O_2^{\cdot-}/H_2O_2$-dependent killing of these two organisms. However, the $HO^{\cdot}$ generated upon exposure of organisms to $O_2^{\cdot-}/H_2O_2$ is likely the result of the catalytic activity of intracellular iron chelates. In contrast, given that MPO is associated with the bacterial cell surface, $HO^{\cdot}$ formation would likely occur in close proximity to the bacterial outer membrane. Thus, the increased killing of these organisms following MPO-preincubation could be in part related to site-specific $HO^{\cdot}$-mediated damage to the bacterial outer membrane.

The simple ability to detect MPO-dependent $HO^{\cdot}$ production in conjunction with the coexistent enhancement of killing of the organisms does not prove a cause and effect relationship. Since the primary product of MPO (HOCl) is microbicidal [1], it is important to attempt to delineate the relative role of MPO-derived HOCl and $HO^{\cdot}$ in MPO-mediated enhancement of bacterial killing. The formation of HOCl by MPO requires only the presence of H_2O_2 and Cl^- [1], whereas the ability of MPO to generate $HO^{\cdot}$ requires the additional presence of $O_2^{\cdot-}$ [7]. In contrast to the results we obtained with xanthine oxidase/lumazine, preincubation with MPO did not enhance the capacity of the H_2O_2 (but not $O_2^{\cdot-}$) forming glucose/glucose oxidase system to kill either *E. coli* or *P. aeruginosa*. Other investigators have failed to detect enhanced killing of MPO-coated organisms including *E. coli*, by H_2O_2 alone [13,46].These results are consistent with a role for MPO-derived $HO^{\cdot}$ in the ability of this enzyme to increase oxidant-mediated killing of these organisms when it is bound to the cell surface. Others have reported that preincubation of other organisms with MPO does enhance their susceptibility to killing by H_2O_2 [18] or decreases the amount of exogenous MPO which had to be present for killing to occur [12,13]. In what may seem contradictory data to ours, Klebanoff failed to detect any clear effect of removing $O_2^{\cdot-}$ with SOD or of $HO^{\cdot}$ scavengers on the ability of an MPO-supplemented xanthine/xanthine oxidase to kill *E. coli* and other organisms [32]. However, in this work the effect of MPO in solution, present in far greater concentrations than in our experiments, rather than only that bound to the organism itself was examined. Under such conditions the amount of HOCl formed is likely much higher and may well be sufficient to kill organisms independent of $HO^{\cdot}$ production.

In spite of evidence pointing to the possible role of MPO-derived $HO^{\cdot}$ in enhanced bacterial killing, our data must still be interpreted cautiously. There is also data that the enzymatic activity of MPO is enhanced by $O_2^{\cdot-}$ [47–50]. We know of no way to eliminate this as an alternate explanation for our observations.

In summary, this report extends the work of others [12,13] indicating that MPO binds to the outer surface of bacteria enhancing their susceptibility to killing by $O_2^{\cdot-}$ and H_2O_2. These events could be important to the microbicidal activity of neutrophils. In addition, it has been suggested that coating of bacteria with neutrophil-derived MPO could also enhance the ability of macrophages, which do not possess this enzyme, to kill these bacteria [51,52]. Although requiring further study, the present data also raise the possibility that MPO – derived $HO^{\cdot}$ in addition to HOCl itself could contribute to this process.

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References

[1] Hurst, J.K. and Barrette, W.C., Jr. (1989) CRC Crit. Rev. Biochem. Mol. Biol. 24, 271–328.
[2] Bielski, B.H.J. and Allen, A.O. (1977) J. Phys. Chem. 81, 1048–1050.

[3] Maxson, C.J., Greenfield, S.M. and Turner, J.L. (1992) Am. J. Gastroenterol. 87, 708–713.
[4] Cohen, M.S., Britigan, B.E., Hassett, D.J. and Rosen, G.M. (1988) Rev. Infect. Dis. 10, 1088–1096.
[5] Weiss, S.J. (1986) Acta Physiol. Scand. (Suppl.) 548, 9–37.
[6] Britigan, B.E., Rosen, G.M., Chai, Y. and Cohen, M.S. (1986) J. Biol. Chem. 261, 4426–4431.
[7] Ramos, C.L., Pou, S., Britigan, B.E., Cohen, M.S. and Rosen, G.M. (1992) J. Biol. Chem. 267, 8307–8312.
[8] Britigan, B.E., Roeder, T.L. and Shasby, D.M. (1992) Blood 79, 699–707.
[9] Gannon, D.E., Varani, J., Phan, S.H., Ward, J.H., Kaplan, J., Till, G.O., Simon, R.H., Ryan, U.S. and Ward, P.A. (1987) Lab. Invest. 57, 37–44.
[10] Britigan, B.E., Hassett, D.J., Rosen, G.M., Hamill, D.R. and Cohen, M.S. (1989) Biochem. J. 264, 447–455.
[11] Winterbourn, C.C. (1986) J. Clin. Invest. 78, 545–550.
[12] Selvaraj, R.J., Zgliczynski, J.M., Paul, B.B. and Sbarra, A.J. (1978) J. Infect. Dis. 137, 481–485.
[13] Miyasaki, K.T., Zambon, J.J., Jones, C.A. and Wilson, M.E. (1987) Infect. Immunol. 55, 1029–1036.
[14] Klebanoff, S.J. and Clark, R.A. (1978) The Neutrophil: Function and Clinical Disorders, pp. 347, Elsevier/North Holland Biomedical Press, New York.
[15] Esaguy, N., Aguas, A.P. and Silva, M.T. (1989) J. Leukocyte Biol. 46, 51–62.
[16] Locksley, R.M., Wilson, C.B. and Klebanoff, S.J. (1982) J. Clin. Invest. 69, 1099–1111.
[17] Wright, C.D. and Nelson, R.D. (1988) Biochem. Biophys. Res. Commun. 154, 809–817.
[18] Ramsey, P.G., Martin, T., Chi, E. and Klebanoff, S.J. (1982) J. Immunol. 128, 415–420.
[19] Pruitt, K.M., Adamson, M. and Arnold, R. (1979) Infect. Immunol. 25, 304–309.
[20] Klebanoff, S.J. and Clark, R.A. (1975) Blood 45, 699–707.
[21] Buettner, G.R. (1988) J. Biochem. Biophys. Methods 16, 27–40.
[22] Britigan, B.E., Pou, S., Rosen, G.M., Lilleg, D.M. and Buettner, G.R. (1990) J. Biol. Chem. 265, 17533–17538.
[23] Sun, Y., Elwell, J.H. and Oberley, L.W. (1988) Free Radic. Res. Commun. 5, 67–75.
[24] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[25] Buettner, G.R. and Chamulitrat, W. (1990) Free Radic. Biol. Med. 8, 55–56.
[26] Buettner, G.R. (1990) Free Radic. Res. Commun. 10, 5–9.
[27] Fridovich, I. (1970) J. Biol. Chem. 245, 4053–4057.
[28] Nagano, T. and Fridovich, I. (1985) J. Free Radic. Biol. Med. 1, 39–42.
[29] Babior, B.M., Curnutte, J.T. and Kipnes, R.S. (1975) J. Lab. Clin. Med. 85, 235–244.
[30] Root, R.K. and Metcalf, J.A. (1977) J. Clin. Invest. 60, 1266–1279.
[31] Green, T.R. and Shangguan, X. (1993) J. Biol. Chem. 268, 857–861.
[32] Klebanoff, S.J. (1974) J. Biol. Chem. 249, 3724–3728.
[33] Bynoe, L.A., Pou, S., Gottsch, J.D. and Rosen, G.M. (1991) Biochem. Biophys. Res. Commun. 179, 1305–1310.
[34] Hassett, D.J., Charniga, L., Bean, K., Ohman, D.E. and Cohen, M.S. (1992) Infect. Immun. 60, 328–336.
[35] Engel, P., Trageser, M. and Unden, G. (1991) Arch. Microbiol. 156, 463–470.
[36] Czapski, G. (1984) Isr. J. Chem. 24, 29–32.
[37] Winterbourn, C.C. (1987) Free Radic. Biol. Med. 3, 33–39.
[38] Candeias, L.P., Patel, K.B., Stratford, M.R.L. and Wardman, P. (1993) FEBS Lett. 333, 151–153.
[39] Rosen, H. and Klebanoff, S.J. (1978) J. Exp. Med. 149, 27–39.
[40] Klebanoff, S.J. and Hamon, C.B. (1972) J. Reticuloendothel. Soc. 12, 170–196.
[41] Imlay, J.A. and Linn, S. (1988) Science 240, 1302–1309.
[42] Touati, D. (1991) Free Radic. Res. Commun. 12–13 PT 1, 379–382.
[43] Dallmier, A.W. and Martin, S.E. (1990) Appl. Environ. Microbiol. 56, 2807–2810.
[44] Müller, J. and Janz, S. (1993) Environ. Mol. Mutagen. 22, 157–163.
[45] Hassett, D.J. and Cohen, M.S. (1989) FASEB J. 3, 2574–2582.
[46] Mata, L., Castillo, H., Sánchez, L., Puyol, P. and Calvo, M. (1994) J. Dairy Res. 61, 427–432.
[47] Kettle, A.J. and Winterbourn, C.C. (1990) Biochim. Biophys. Acta 1052, 379–385.
[48] Kettle, A.J. and Winterbourn, C.C. (1991) Free Radic. Res. Commun. 12–13 PT 1, 47–52.
[49] Kettle, A.J. and Winterbourn, C.C. (1989) Biochem. J. 263, 823–828.
[50] Kettle, A.J. and Winterbourn, C.C. (1988) Biochem. J. 252, 529–536.
[51] Shepherd, V.L. and Hoidal, J.R. (1990) Am. J. Respir. Cell Mol. Biol. 2, 335–340.
[52] Leung, K.-P. and Goren, M.B. (1989) Cell Tissue Res. 257, 653–656.