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Comparative study of HOCl-inflicted damage to bacterial DNA *ex vivo* and within cells

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ABSTRACT

The prospects for using bacterial DNA as an intrinsic probe for HOCl and secondary oxidants/chlorinating agents associated with it has been evaluated using both *in vitro* and *in vivo* studies. Single-strand and double-strand breaks occurred in bare plasmid DNA that had been exposed to high levels of HOCl, although these reactions were very inefficient compared to polynucleotide chain cleavage caused by the $OH^{\cdot}$-generating reagent, peroxynitrite. Plasmid nicking was not increased when intact *Escherichia coli* were exposed to HOCl; rather, the amount of recoverable plasmid diminished in a dose-dependent manner. At concentration levels of HOCl exceeding lethal doses, genomic bacterial DNA underwent extensive fragmentation and the amount of precipitable DNA–protein complexes increased several-fold. The 5-chlorocytosine content of plasmid and genomic DNA isolated from HOCl-exposed *E. coli* was also slightly elevated above controls, as measured by mass spectrometry of the deaminated product, 5-chlorouracil. However, the yields were not dose-dependent over the bactericidal concentration range. Genomic DNA recovered from *E. coli* that had been subjected to phagocytosis by human neutrophils occasionally showed small increases in 5-chlorocytosine content when compared to analogous cellular reactions where myeloperoxidase activity was inhibited by azide ion. Overall, the amount of isolable 5-chlorouracil from the HOCl-exposed bacterial cells was far less than the damage manifested in polynucleotide bond cleavage and cross-linking.

Introduction

One recurrent issue concerning the biochemistry of host cellular defense mechanisms is the relative extent to which oxidative and nonoxidative processes contribute to microbial killing within phagocytes. Current opinion on this subject varies widely; for example, several groups have presented evidence supporting the notion that myeloperoxidase (MPO)[1]-generated hypochlorous acid (HOCl) is a primary neutrophil microbicide [1–4], whereas others have disputed this evidence, and suggested that MPO functions physiologically as a catalase [5], thereby protecting bactericidal hydrolytic enzymes introduced during phagocytosis from inactivation by respiration-generated H_2O_2. Evidence also exists for an additional type of nonoxidative mechanism involving other granule-derived antimicrobial proteins that function by disrupting bacterial homeostasis without inactivating specific enzymes [6,7]. Although one might surmise from the very different nature of the chemistries comprising oxidative versus nonoxidative killing that distinguishing between them should be straightforward, the problem is convoluted by their simultaneous participation in intraphagosomal reactions [8]. Thus, for example, the demonstration that neutrophils kill bacteria under conditions where oxidative processes are inhibited cannot be taken as evidence that oxidative mechanisms do not exist. Furthermore, rather than being redundant, the distinct microbicidal mechanisms are likely to be synergistic [4,9,10].

To evaluate the role of HOCl in intraphagosomal killing by neutrophils, one needs to know both the extent of its formation by MPO-catalyzed oxidation of chloride ion and how its subsequent reactions with bacterial and host-derived targets are distributed. With respect to the first question, a recent kinetic modeling study based upon experimentally determined kinetic parameters and phagosomal dimensions predicts that ~90% of the O_2 consumed by stimulated neutrophils will be converted to HOCl [11]. In developing the model it was recognized, but not explicitly taken into account, that Cl^- could be the limiting intraphagosomal reagent, a condition which would lower the predicted yields. This possibility arises because the amount of extracellular fluid introduced during phagocytosis is very small, hence the amount of Cl^- entrapped during formation of the phagosome is less than the amount of O_2 consumed by NADPH oxidase-dependent stimulated respiration. Since the $HOCl/O_2$ stoichiometry cannot exceed 1:1 in the coupled NADPH oxidase/MPO catalyzed reaction, i.e., $2H^+ + NAD$-

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[1] *Abbreviations used*: BCS, bathocuproine sulfonate; GFP, green fluorescent protein; HBSS, Hanks balanced salt solution; LB, Luria broth; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride.

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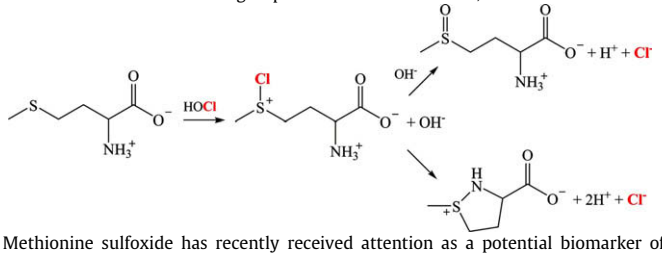
$PH + O_2 + Cl^- \rightarrow NADP^+ + HOCl + H_2O$, chloride ion will be limiting unless it is replenished in subsequent reactions of HOCl or by transmembrane ion transport processes [12–13]. Chemical trapping studies in which chloride is incorporated into stable reaction products are consistent with this viewpoint. Specifically, in studies using fluorescein-conjugated particles as bacterial mimics, it was found that the amount of chloride trapped within the phagosome as mono-, di-, and trichlorofluoresceins corresponded closely to the calculated amount of extracellular Cl^- introduced during its formation [3].[2] This amount, however, was only ~10% of the O_2 consumed during stimulated respiration. In contrast, when soluble agonists were used to trigger extracellular release of MPO, as much as 40% of the O_2 consumed could be trapped as taurine chloramine [16]. Under these conditions, where reaction occurs in the extracellular milieu, Cl^- is present in large excess and cannot be significantly depleted over the course of the neutrophil respiratory burst.

The chlorofluorescein trapping and O_2 consumption measurements indicate that between $\sim10^8$ and $\sim10^9$ HOCl molecules per entrapped particle can be generated within phagosomes [3,11]. The lower limit is clearly a conservative estimate because the phagosomal milieu contains numerous targets that react more rapidly than fluorescein with HOCl [17–20]. Prominent among these are biological sulfhydryl and thioether compounds that, unlike fluorescein, undergo net oxidation with regeneration of Cl^- as one of the reaction products, which then is available for MPO-catalyzed formation of additional HOCl.[3]

Attempts to assess the intraphagosomal *distribution* of HOCl reactions have made use of ^{13}C-labeling [25,26] to determine the relative extent of chlorination of bacterial and host-derived protein tyrosyl groups. Almost all (~94%) of the HOCl consumed in this reaction was trapped by tyrosyl groups on the host cell protein [25], which was primarily a consequence of its greater abundance within the phagosome. Nonetheless, if ~90% of the O_2 consumed is converted to HOCl, as implied by the kinetic model [11], then it follows that each bacterium is exposed to $\sim10^8$ HOCl molecules, a value which is consistent with the fluorescein trapping results [3]. This number is provocative because the amount of HOCl required to kill various strains of bacteria in *in vitro* assays is also $\sim10^8$ HOCl/bacterium [3,27,28]. Thus, one might infer that bactericidal amounts of HOCl are generated within neutrophil phagosomes.

[2] Based upon estimates from electron micrographs [14], the average diameter of a neutrophil phagosome is ~1.5 μm. If one assumes spherical geometry, the corresponding volume of a phagosome containing a 1.0 μm diameter particle is $\sim1.2 \times 10^{-12}$ mL; $\sim10^8$ chlorine atoms trapped by the particle corresponds to $\sim1.7 \times 10^{-13}$ mmols Cl^-, requiring an apparent intraphagosomal concentration of ~0.14 M. The measured concentration of Cl^- occluded within the phagosomes of human neutrophils scales linearly with the extracellular concentration [15]; at the concentration levels present in serum, the levels extrapolated from these data would be ~0.1 M.

[3] A pertinent example is methionine, which is rapidly oxidized by HOCl to give as products methionine sulfoxide [22] and dehydromethionine [21], a cyclized compound containing a covalent bond between the methionyl sulfur and amido nitrogen atoms. These reactions are thought to proceed via intermediary formation of a chlorosulfonium cation [23], which subsequently undergoes nucleophilic attack by either solvent or the α-amino group with release of chloride, as illustrated below:



Methionine sulfoxide has recently received attention as a potential biomarker of intraphagosomal bactericidal reactions involving neutrophil-generated HOCl [24].

Although these studies appear to be mutually self-consistent, there are several indeterminate factors which might impact significantly upon the conclusions. Tyrosine chlorination is a relatively slow reaction [11] and is unlikely to be involved in bacterial killing, which appears to involve very rapidly reacting sites on the bacterial cell envelope [18,29,30]. Furthermore, MPO strongly associates with the bacterial wall [31–33], suggesting that it could direct HOCl toward vulnerable bacterial targets in the phagosome. Kinetic modeling studies (Cape, unpublished research) suggest that this proximity effect would be important for targets whose bimolecular rate constants exceed $10^5\ M^{-1}\ s^{-1}$; these include, for example, cysteine sulfhydryl and methionyl thioether groups and iron-sulfur clusters. Finally, HOCl generation within the phagosome is much slower than the microbicidal reactions following *in vitro* bolus additions of the oxidant [18,34], so that protective responses to oxidative stress engendered in bacteria undergoing phagocytosis [35,36] could render them less susceptible to oxidative killing than implied by the *in vitro* bactericidal assays. Having available alternative bacterial components as selective probes for HOCl, particularly ones more reactive than tyrosine, could be very useful in describing the events comprising bactericidal action in neutrophils [24]. In the present study, we have considered using both plasmid and genomic bacterial DNA as potential probes. For reasons discussed herein, our results suggest that monitoring polynucleotide strand cleavage or formation of 5-chlorouracil, a stable product of the reaction between HOCl and cytosine, will not prove useful in this capacity; however, the data obtained suggest that DNA–protein cross-linking could be developed as an intrinsic probe. Indeed, the dramatic increase in DNA–protein complexation following exposure to lethal levels of HOCl was unanticipated, and constitutes the most remarkable finding of these studies.

Materials and methods

Materials

The following chemicals and biological materials were obtained from the indicated sources: alkaline phosphatase (Roche Applied Science); ethylenediaminetetraacetic acid (EDTA), nuclease S_1, proteinase K, puromycin, sodium thiosulfate, and thymidine phosphorylase (Sigma); nuclease P_1 (U.S. Biological); Hoechst 33342 trihydrochloride trihydrate (Molecular Probes); pETBlue plasmid and NovaBlue cells (Novagen). Hypochlorous acid (HOCl) was purified by vacuum distillation of commercial bleach solutions which had been neutralized to pH 7–7.5 by addition of phosphoric acid [37]; reagent concentrations were determined spectrophotometrically as the OCl^- ion ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ [38]). Chloramine (NH_2Cl) was prepared by flow-mixing equal volumes of 0.1 M NH_3 in water with 8.0 mM HOCl in 50 mM phosphate buffer, pH 7.4 through a tangential 12-jet mixer; the spectrophotometrically determined NH_2Cl yield was 95% (3.8 mM), based upon a reported value of $\varepsilon_{242} = 429\ M^{-1}\ cm^{-1}$ [39]. Peroxynitrous acid (ONOOH) was prepared as alkaline solutions of the sodium salt by a quenched flow-mixing procedure involving reaction between nitrous acid and hydrogen peroxide [40]; reagent concentrations, determined in 10 mM NaOH as the $ONOO^-$ anion ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$ [41]), were typically ~100 mM and contained ⩽20% residual nitrite ion. Other chemicals were best available grade from commercial sources and used as received; except where indicated, reverse osmosis–deioinized water was used in the preparation of reagent solutions.

Two cell lines of *Escherichia coli*, ATCC 11775 and the engineered K-12 strain, NovaBlue, were used to study bacterial genomic and plasmid DNA damage caused by exposure to oxidants (HOCl, ONOOH) or upon phagocytosis by human neutrophils. Most