Superoxide radicals can act synergistically with hypochlorite to induce damage to proteins

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Abstract Activated phagocytes generate both superoxide radicals via a respiratory burst, and HOCl via the concurrent release of the haem enzyme myeloperoxidase. Amine and amide functions on proteins and carbohydrates are major targets for HOCl, generating chloramines (RNHCl) and chloramides (RC(O)NClR′), which can accumulate to high concentrations (> 100 μM). Here we show that superoxide radicals catalyse the decomposition of chloramines and chloramides to reactive nitrogen-centred radicals, and increase the extent of protein fragmentation compared to that observed with either superoxide radicals or HOCl, alone. This synergistic action may be of significance at sites of inflammation, where both superoxide radicals and chloramines/chloramides are formed simultaneously.

Key words: Hypochlorite; Superoxide radical; Chloramine; Nitrogen-centred radical; Myeloperoxidase; Protein oxidation

1. Introduction

Activation of phagocyte cells both in vivo and in vitro results in the generation of $O_2^{-\bullet}$ and H_2O_2 and the release of the haem enzyme myeloperoxidase (MPO). MPO catalyses the reaction of H_2O_2 with Cl^- ions to give HOCl, which is a major bactericide [1–3]. Excessive or misplaced generation of this oxidant can, however, damage neighbouring cells and this is believed to be important in certain human pathologies (e.g. arthritis, some cancers and atherosclerosis) [4,5].

HOCl reacts rapidly with many biological molecules including DNA, proteins, lipids, cholesterol and polysaccharides [6–12]. Proteins are major targets due to their abundance and high rate constants for reaction, and this can result in side chain damage, fragmentation, aggregation, and an increased susceptibility to proteolytic degradation [6,11,13–16]. Though amine and imine functions (e.g. N-terminus, Lys, His and Trp side chains) are less reactive than Cys and Met residues [6,16], they are major targets for HOCl as a result of their high abundance. Reaction with amines gives chloramines (RNHCl) [17], whereas amides (e.g. peptide bonds) yield chloramides (RC(O)NClR′ species) via a slower reaction [16]. The latter are important intermediates in protein backbone fragmentation [11,18]. Chloramines and chloramides have, unlike HOCl or H_2O_2, long biological half-lives (minutes–hours) and can accumulate (cf. detection of > 100 μM taurine chloramine on incubation of 2×10^6/ml activated neutrophils over 60 min [3]). Thermal, metal ion-catalysed, and UV-induced decomposition of chloramines/chloramides yields reactive nitrogen-centred radicals via N–Cl bond cleavage (reactions 1 and 2), and carbonyl groups [11,18,19].

$$\mathrm{RNHCl} + \mathrm{heat/UV\ light} \rightarrow \mathrm{RNH}^{\bullet} + \mathrm{Cl}^{\bullet} \quad (1)$$

$$\mathrm{RNHCl} + \mathrm{Fe}^{2+} \rightarrow \mathrm{RNH}^{\bullet} + \mathrm{Cl}^{-} + \mathrm{Fe}^{3+} \quad (2)$$

In this study we examine the hypothesis that $O_2^{-\bullet}$ might stimulate radical formation, via reaction 3, as $O_2^{-\bullet}$ is generated concurrently with HOCl, and hence chloramines and chloramides, in many biological situations. This reaction should be favourable, as N–Cl species are powerful oxidants, and the N–Cl bond is weak [20]. Reaction 3 is analogous to the known $O_2^{-\bullet}$-mediated reduction of HOCl [21,22].

$$\mathrm{RNHCl} + \mathrm{O}_2^{-\bullet} \rightarrow \mathrm{RNH}^{\bullet} + \mathrm{Cl}^{-} + \mathrm{O}_2 \quad (3)$$

2. Materials and methods

2.1. Materials

Solutions were prepared using water filtered through a four-stage Milli Q system. pH control was achieved using 0.1 M pH 7.4 phosphate buffer treated with Chelex resin (Bio-Rad, Hercules, CA, USA) to remove any contaminating trace metal ions. Enzymes, proteins, and amino acids were obtained from Sigma (St. Louis, MO, USA) and used as supplied. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO; ICN, Seven Hills, NSW, Australia) was purified before use by treatment with activated charcoal. HOCl solutions were prepared by dilution of a concentrated stock (0.5 M in 0.1 M NaOH) into 0.1 M pH 7.4 phosphate buffer, with the HOCl concentration determined spectrophotometrically at pH 12 ($\varepsilon_{292\,nm}$ 350 M^{-1} cm^{-1}). Di-(4-carboxybenzyl)hyponitrite (SOTS-1) [23] was a gift from Prof. Chris Easton (Research School of Chemistry, Canberra, Australia). Superoxide dismutase (SOD) was obtained from Sigma (from bovine erythrocytes, S-2515, St. Louis, MO, USA). All other chemicals were of analytical reagent grade. 5-Thio-2-nitrobenzoic acid (TNB; 35–45 μM) was used to assess chloramine concentrations after 15 min reaction using $\varepsilon_{412\,nm}$ 13 600 M^{-1} cm^{-1} [11,17]. TNB was added to the samples after incubation with $O_2^{-\bullet}$ at 37°C for the appropriate time. Neither the SOTS-1 nor any components of the acetaldehyde (ethanal, CH_3CHO)–xanthine oxidase (XO) $O_2^{-\bullet}$ generating systems interfered with the TNB

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Abbreviations: CH_3CHO, acetaldehyde (ethanal); DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; HOCl, the physiological mixture of hypochlorous acid and its anion ^-OCl; BSA, bovine serum albumin; SOTS-1, di-(4-carboxybenzyl)hyponitrite; TNB, 5-thio-2-nitrobenzoic acid; XO, xanthine oxidase

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assay. Thus, identical chloramine concentrations were detected in the $t=0$ samples of HOCl-treated taurine and bovine serum albumin (BSA) assayed either in the presence or absence of the SOTS-1 or CH_3CHO–XO system. Similarly, decomposed SOTS-1 had no effect on the assay.

2.2. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER4103TM cavity. Samples were contained in a flattened, aqueous-sample cell (WG-813-SQ; Wilmad, Buena, NJ, USA) and spectral recording was initiated within 2 min of the addition of the spin trap. The spin trap was added to the reaction 2–60 min after the HOCl to avoid direct reaction of HOCl with DMPO [24]. Hyperfine couplings were measured directly from the field scan and confirmed by computer simulation (WINSIM, available at www.epr.niehs.nih.gov). Correlation coefficients between simulated and experimental spectra were >0.95. Typical EPR spectrometer settings were: gain, 1×10^5–10^6; modulation amplitude, 0.01–0.05 mT; time constant, 0.16 s; scan time, 84 s; resolution, 1024 points; centre field, 348 mT; field scan, 8–10 mT; power, 25 mW; frequency, 9.76 GHz; four scans averaged.

2.3. Protein electrophoresis

Proteins were separated by the method of Laemmli using 8% polyacrylamide gels [11]. Protein samples were added to an equal volume of 60 mM Tris–HCl buffer pH 6.8 containing glycerol (10% v/v), 2-mercaptoethanol (5% v/v), SDS (2% w/v) and bromophenol blue (0.01% w/v). Samples were reduced at 95°C before loading onto the gel. Bands were visualised using Coomassie blue staining. Gels were scanned and digitised over a linear range using a Bio-Rad Gel Doc 1000 system and software (Bio-Rad, Hercules, CA, USA).

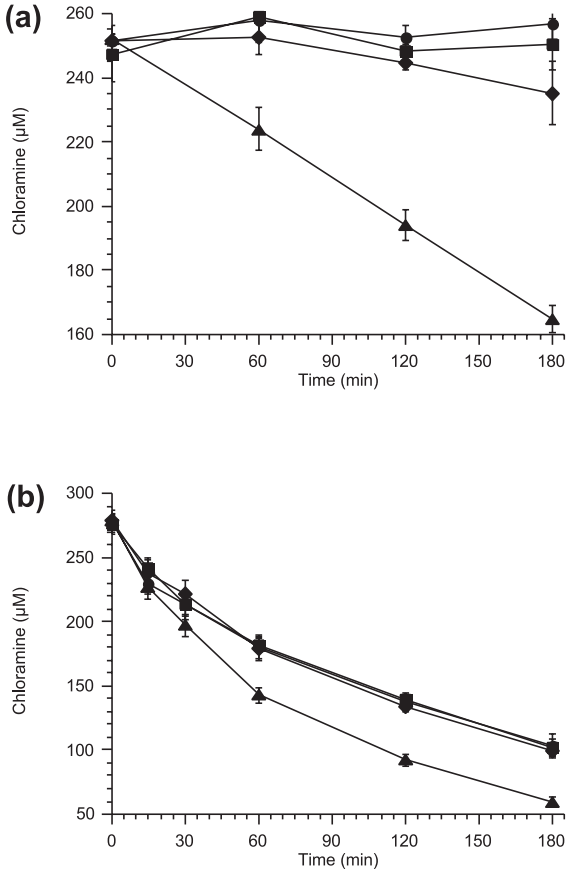

Fig. 1. Decay of chloramines over time in the presence, and absence, of an $O_2^{-\bullet}$ generating source. (a) Taurine (1.25 mM) was incubated for 2 min with HOCl (250 μM), and (b) BSA (10 μM) was incubated for 5 min with HOCl (500 μM) at 4°C, then in each case SOTS-1 (1 mM) (triangle), with SOD (100 U) (diamond) or decomposed SOTS-1 (1 mM) (square) or buffer (circle) was added and the samples incubated at 37°C. Data are mean ± S.D. for four experiments.

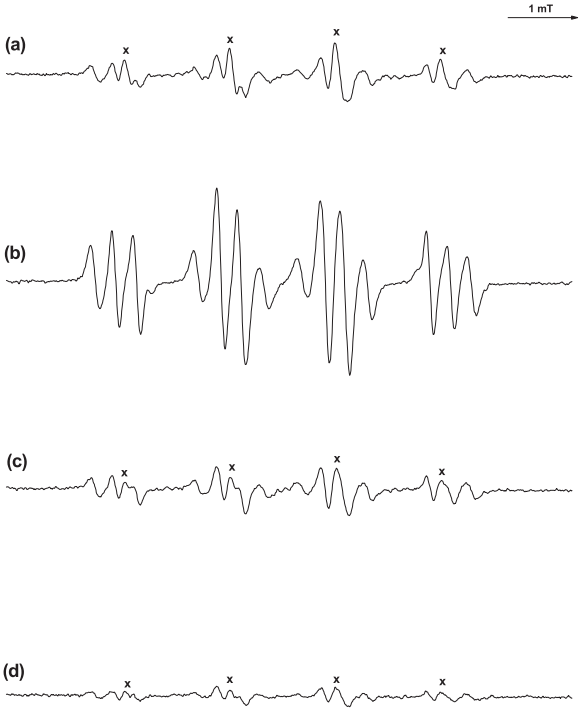

Fig. 2. EPR spectra observed on reaction of taurine with HOCl in the absence, or presence, of $O_2^{-\bullet}$. Taurine (25 mM) was incubated for 2 min with HOCl (5 mM) at room temperature then DMPO (100 mM), CH_3CHO (2.5 mM) and XO (0.25 U) were added. The mixture was then examined by EPR spectroscopy with the recording of spectra starting within 90 s of the final addition. (a) With no added CH_3CHO or XO. (b) Complete system. (c) As (b), but with no CH_3CHO. (d) As (b), but with no XO. Signals marked X attributed to the DMPO-OH adduct. Non-marked signals assigned to a taurine-derived, nitrogen-centred radical adduct with a(N) 1.47, a(H) 1.80, a(N) 0.29 mT.

3. Results

3.1. Stability of chloramines in the presence of $O_2^{-\bullet}$

Chloramines were generated on taurine (1.25 mM), and BSA (10 μM) by incubation with HOCl (250 and 500 μM) for 2 and 5 min respectively at 4°C. The stability of the chloramines was investigated at 37°C using the TNB assay, in either the absence or presence of a source of $O_2^{-\bullet}$, with the $O_2^{-\bullet}$ generated either by the thermal decomposition of SOTS-1 in the presence of O_2 ($t_{1/2}$ 81 min [23]), or an aerobic CH_3CHO–XO system. Both $O_2^{-\bullet}$ generating systems promoted the decay of the chloramines (Fig. 1). The extent of stimulation varied with the species examined, with greater loss observed with the more stable species (e.g. taurine chloramine). Enhanced rates of chloramine decay were not detected using the SOTS-1 $O_2^{-\bullet}$ system in the presence of SOD (100 U), or when decomposed SOTS-1 solutions were employed (Fig. 1). The stimulation of chloramine decomposition observed with the aerobic CH_3CHO–XO system was incompletely ameliorated by SOD (1000 U), as XO alone had a minor stimulatory effect (data not shown). The latter is attributed to the presence of contaminating metal ions in the XO preparation which are notoriously hard to remove (e.g. [25]).

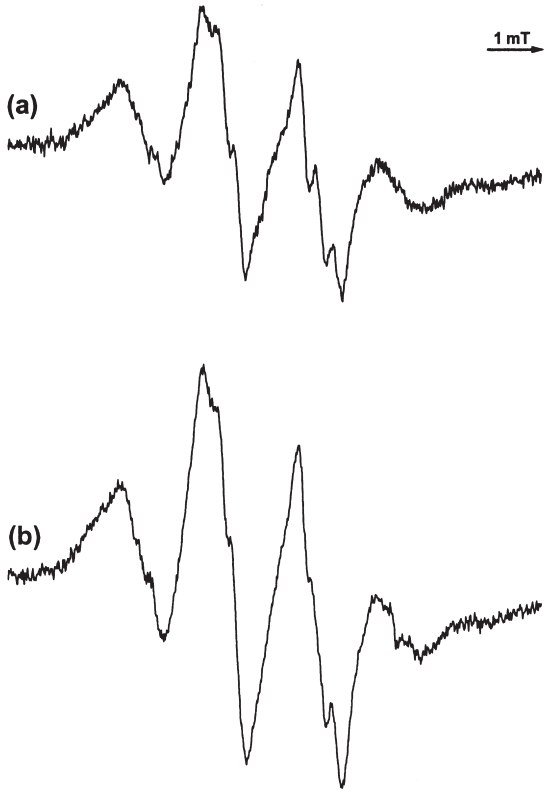

Fig. 3. EPR spectra observed on reaction of BSA with HOCl in the absence, or presence, of $O_2^{-\bullet}$. BSA (1.05 mM) was incubated for 5 min with HOCl (5 mM) at room temperature then DMPO (200 mM), CH_3CHO (10 mM) and XO (1 U) were added. The mixture was then examined by EPR spectroscopy with the recording of spectra starting within 90 s of the final addition. (a) With no CH_3CHO and XO, (b) complete system. Broad signals in both spectra assigned to a protein-derived, nitrogen-centred, radical adduct with *a*(N) 1.50, *a*(H) 1.79, *a*(N) 0.29 mT as reported previously [11].

3.2. Formation of nitrogen-centred radicals from chloramines in the presence of $O_2^{-\bullet}$

The effect of $O_2^{-\bullet}$ on radical formation from taurine and BSA-derived chloramines was investigated using the aerobic CH_3CHO–XO system and EPR spin trapping using DMPO. SOTS-1 was not employed in this case due to the slow rate of $O_2^{-\bullet}$ formation from this compound [23] compared to the half-life of the spin adducts. Incubation of the HOCl-treated taurine and BSA in the presence of $O_2^{-\bullet}$ and DMPO resulted in an increase in the intensity of the nitrogen-centred radical adduct signals, compared to signals observed in the absence of $O_2^{-\bullet}$ (Figs. 2 and 3). Stimulation of radical formation was also observed using other spin traps to assess radical generation, and with a range of other chloramines and chloramides, including those generated, by HOCl, on free Lys (using either DMPO or PBN as spin trap), *N*-acetyl His (with DEPMPO as trap), *N*-acetyl sugars, ethyl guanidine sulphate, *N*-acetyl-Arg-OMe, acetamidine, *N*-ethyl-acetamide, *N*-*t*-butyl-acetamide (all with DMPO as trap). The extent of stimulation of radical formation was greatest for the most stable chloramines and chloramides, as these show the slowest uncatalysed decay. Omission of either CH_3CHO or XO markedly diminished this stimulation (Fig. 2). Addition of SOD (200 U) also resulted in a significant reduction (25%) in the concentration of the taurine radical adduct observed with the CH_3CHO–XO system, and similar, or greater, extents of protection were seen with other chloramines. XO alone gave a small increase in signal intensity (Fig. 2) due to the presence of trace transition metal ions [25]. No substrate-derived signals were detected on reaction of native taurine or BSA with the complete CH_3CHO–XO system.

Signals from the well-characterised DMPO-OH and DMPO-OOH adducts were also detected [26] with the complete aerobic CH_3CHO–XO system. This is attributed to competition between the chloramine and spin trap for $O_2^{-\bullet}$. The DMPO-OH and DMPO-OOH signals were more intense in the absence of the chloramine/chloramide, and decreased in intensity with increasing concentrations of these species. An estimate of the rate constant for reaction of chloramines with $O_2^{-\bullet}$ (reaction 3) can be obtained from the concentration required to remove 50% of the DMPO-OOH signal at a fixed concentration of DMPO given that the rate constant for the trapping of $O_2^{-\bullet}$ by DMPO is ca. 10 $dm^3\ mol^{-1}\ s^{-1}$ [27]. With taurine chloramine this gives a rate constant of ca. $5–6\times10^2$ $dm^3\ mol^{-1}\ s^{-1}$.

3.3. Fragmentation of HOCl-treated proteins in the presence of $O_2^{-\bullet}$

The effect of $O_2^{-\bullet}$ on the loss of the parent BSA protein, as determined by SDS–PAGE, was investigated by incubation of BSA-derived chloramines (10 μM BSA/500 μM HOCl) in the absence and presence of SOTS-1 (1.5 mM) for 1, 2 or 4 h at 37°C. Enhanced loss of the parent protein band was observed in the presence of SOTS-1 compared to control samples containing BSA chloramines alone (Fig. 4). This stimulation was prevented by the presence of SOD (100 U). Similar stimulation was observed using the aerobic CH_3CHO–XO system (data not shown). No change in the BSA protein band was detected on incubation of native BSA with either SOTS-1 or CH_3CHO–XO at 37°C (data not shown) [11].

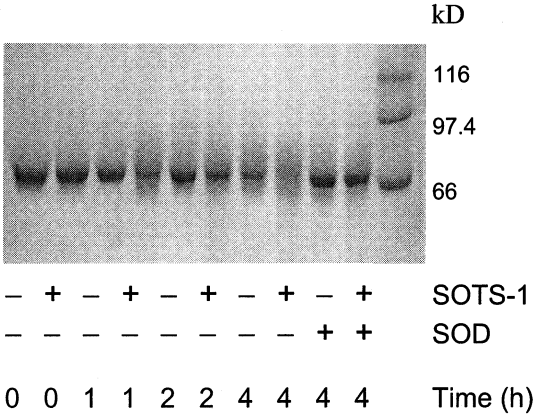

Fig. 4. Degradation of HOCl-treated BSA in the absence, or presence, of $O_2^{-\bullet}$. BSA (10 μM) was treated with HOCl (500 μM) at 4°C for 5 min, then incubated at 37°C for 1, 2 or 4 h with SOTS-1 (1.5 mM) or buffer, in the presence or absence of SOD (100 U) before quenching with methionine (20 mM) to remove remaining chloramines. Samples were subsequently subjected to SDS–PAGE [11].

4. Discussion

Previous studies have shown that protein-derived chloramines can undergo thermal decay to give radicals that have been postulated to be important intermediates in HOCl-mediated protein degradation [11,18]. The rates of these reactions are enhanced by the presence of certain metal ions (e.g. Fe^{2+} and Cu^{+}), and UV light [28].

In this study we have shown that the decay of chloramines and chloramides is stimulated by $O_2^{-\bullet}$, and that this gives rise to increased yields of nitrogen-centred radicals (via reaction 3), and enhanced degradation of HOCl-treated proteins. All of these processes are chloramine dependent, and are inhibited by SOD. The extent of stimulation of each process (chloramine decay, radical formation, polymer degradation) is most marked with the most stable chloramines (e.g. taurine chloramine) and chloramides as these have the slowest rate of non-catalysed decomposition. Minor stimulation was observed with XO alone, with this effect attributed to the presence of contaminating trace metal ions in the enzyme preparation (e.g. [25]). The inhibitory effect of SOD shows that the majority of the observed stimulation arises via $O_2^{-\bullet}$ generation. It is unlikely that the enhanced chloramine decomposition is due to $O_2^{-\bullet}$-catalysed reduction of trace transition metal ions which subsequently interact with the chloramines (cf. previous evidence for transition metal ion catalysis of this process [19,28]) as Chelex treatment has no effect on these reactions. However, this possibility cannot be completely excluded.

$O_2^{-\bullet}$ is generated by a number of cell types and pathways at sites of inflammation where HOCl is also formed. In addition to arising from the oxidative burst of phagocytes, $O_2^{-\bullet}$ is also formed by elimination reactions of α-hydroxyalkyl peroxyl radicals (e.g. those formed by hydrogen atom abstraction from sugars, polysaccharides, the sugar-phosphate backbone of DNA, and Ser/Thr residues on proteins), from α-aminoalkyl peroxyl radicals (e.g. by hydrogen atom abstraction from amino sugars, and Lys residues on proteins), from protein backbone α-carbon peroxyl radicals, from aromatic side chain peroxyl radicals on proteins, from DNA base peroxyl radicals, as well as via direct reaction of O_2 with electrons released from electron transport chains and enzymes [4,29,30]. The prevalence of this radical, and the accumulation of chloramines/chloramides at >100 μM concentrations at sites of inflammation [3] make the synergistic reactions reported here a likely event in both in vitro and in vivo situations. The rate constant obtained for reaction of taurine chloramine with $O_2^{-\bullet}$, and the above data on chloramine/chloramide concentrations suggest that the reaction between these two species will become competitive with dismutation of $O_2^{-\bullet}$, at fluxes of $O_2^{-\bullet}$ which are $<$ca. 10^{-7} M. This is biologically realistic. The occurrence of this synergistic reaction has not been reported previously with stimulated phagocytes, as the determination of chloramine yields only provides a steady-state measurement, rather than total throughput. This $O_2^{-\bullet}$-induced decay of chloramines can result in an underestimation of the yield of HOCl generated by activated phagocytes, as these have often been measured on the assumption that the chloramines formed with, for example, taurine are stable.

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