Human Neutrophils Employ Chlorine Gas as an Oxidant during Phagocytosis

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Abstract

Reactive oxidants generated by phagocytes are of central importance in host defenses, tumor surveillance, and inflammation. One important pathway involves the generation of potent halogenating agents by the myeloperoxidase–hydrogen peroxide–chloride system. The chlorinating intermediate in these reactions is generally believed to be HOCl or its conjugate base, ClO^-. However, HOCl is also in equilibrium with Cl_2, raising the possibility that Cl_2 executes oxidation/halogenation reactions that have previously been attributed to HOCl/ClO^-. In this study gas chromatography–mass spectrometric analysis of head space gas revealed that the complete myeloperoxidase–hydrogen peroxide–chloride system generated Cl_2. In vitro studies demonstrated that chlorination of the aromatic ring of free L-tyrosine was mediated by Cl_2 and not by HOCl/ClO^-. Thus, 3-chlorotyrosine serves as a specific marker for Cl_2-dependent oxidation of free L-tyrosine. Phagocytosis of L-tyrosine encapsulated in immunoglobulin- and complement-coated sheep red blood cells resulted in the generation of 3-chlorotyrosine. Moreover, activation of human neutrophils adherent to a L-tyrosine coated glass surface also stimulated 3-chlorotyrosine formation. Thus, in two independent models of phagocytosis human neutrophils convert L-tyrosine to 3-chlorotyrosine, indicating that a Cl_2-like oxidant is generated in the phagolysosome. In both models, synthesis of 3-chlorotyrosine was inhibited by heme poisons and the peroxide scavenger catalase, implicating the myeloperoxidase-hydrogen peroxide system in the reaction. Collectively, these results demonstrate that myeloperoxidase generates Cl_2 and that human neutrophils use an oxidant with characteristics identical to those of Cl_2 during phagocytosis. Moreover, our observations suggest that phagocytes exploit the chlorinating properties of Cl_2 to execute oxidative and cytotoxic reactions at sites of inflammation and vascular disease. (*J. Clin. Invest.* 1996. 98:1283–1289.) **Key words: myeloperoxidase • inflammation • oxidation • hypochlorous acid • atherosclerosis**

Introduction

Reactive oxidants generated by phagocytes are of central importance in host defenses, tumor surveillance, and inflammation (1–8). When phagocytes become activated, they generate superoxide, which subsequently dismutates to form hydrogen peroxide (H_2O_2). Myeloperoxidase, a heme protein secreted by phagocytes, amplifies the oxidative potential of H_2O_2 by generating cytotoxic oxidants and diffusible radical species (1–8). These oxidants play a critical role in the destruction of invading pathogens (9). However, reactive intermediates generated by activated phagocytes are also potentially deleterious and have been implicated in the pathogenesis of diseases ranging from atherosclerosis to ischemia-reperfussion injury and cancer (4, 6, 8, 10, 11).

The major oxidant generated by the myeloperoxidase-H_2O_2-Cl^- system at physiological concentrations of Cl^- is hypochlorous acid (HOCl)[1] (12–14). The microbicidal and toxic properties of HOCl stem from its chemical reactivity, which includes chlorination of amines (15–17) and unsaturated lipids (18, 19) and oxidative bleaching of heme groups and iron sulfur centers (20). The chlorinating intermediate in these reactions is believed to be HOCl or its conjugate base, hypochlorite (ClO^-; pK_a 7.4; see reference 21). HOCl is also in equilibrium with Cl_2 via a reaction that requires Cl^- and H^+ (pK_a3.3; reference 21):

$$HOC1 + C1^- + H^+ = C1_2 + H_2O \quad (1)$$

The formation of Cl_2 is favored by acidic pH and the presence of Cl^-, suggesting that under these conditions Cl_2 could potentially execute oxidation/halogenation reactions normally ascribed to HOCl/ClO^-. Consonant with this notion, the HOCl-dependent oxidation of a variety of biological compounds that possess extensive π orbital electron systems is augmented by Cl^- and acidic pH (20).

In the current studies we have explored the potential role of Cl_2 as a physiological oxidant. We use gas chromatography-mass spectrometry (GC-MS) to demonstrate that Cl_2 is generated by the myeloperoxidase-H_2O_2-Cl^- system, and we also provide evidence that activated human neutrophils use an oxidant with characteristics identical to those of Cl_2 during phagocytosis.

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1. *Abbreviations used in this paper:* ClO^-, hypochlorite; GC-MS, gas chromatography-mass spectrometry; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; m/z, mass-to-charge ratio; NaOCl, sodium hypochlorite; NCI, negative-ion chemical ionization; pHA, p-hydroxyphenylacetaldehyde; TFA, trifluoroacetic acid.

Methods

Materials

Sodium hypochlorite, H_2O_2, organic solvents and sodium phosphate were obtained from Fisher Chemical Company (St. Louis, MO). Chelex-100 resin and catalase (bovine liver, thymol-free) were from Bio-Rad Laboratories, Inc. (Hercules, CA) and Boehringer Mannheim Biochemicals (Indianapolis, IN), respectively. Hank's Balanced Salt Solution and phosphate buffered saline were obtained from GIBCO-BRL Products (Gaithersburg, MD). Stable isotopes were obtained from Cambridge Isotope Laboratories (Andover, MA). All other materials were purchased from Sigma Chemical Company (St. Louis, MO) except where indicated.

Methods

Isolation of myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7). Human leukocytes obtained by leukopheresis were used as starting material for preparation of myeloperoxidase. The enzyme was isolated by sequential lectin affinity and size exclusion chromatographies as previously described (22, 23). Purified myeloperoxidase (A_{430}/A_{280} ratio of 0.6) was dialyzed against water and stored in 50% glycerol at −20°C. Enzyme concentration was determined spectrophotometrically ($\epsilon_{430} = 170\ mM^{-1}cm^{-1}$; reference 24).

Preparation of Chloride-free Sodium Hypochlorite. Chloride-free sodium hypochlorite (NaOCl) was prepared by a modification of previously described methods (25). Reagent NaOCl (100 ml) mixed with ethyl acetate (100 ml) was protonated by drop-wise addition of concentrated phosphoric acid (final pH $\leq$ 6) with constant agitation. The organic phase containing HOCl was washed twice with H_2O, and HOCl was re-extracted into H_2O by the drop-wise addition of NaOH (final pH $\geq$ 9). Residual ethyl acetate in the aqueous solution of chloride-free NaOCl was removed by bubbling with N_2. The concentration of NaOCl was determined spectrophotometrically ($\epsilon_{292} = 350\ M^{-1}cm^{-1}$; reference 26).

Oxidation of L-tyrosine. All reactions were performed in gas-tight vials and initiated by addition of oxidant (H_2O_2, Cl_2, or $HOCl/ClO^-$) through septa with a gas-tight syringe. The pH dependence of 3-chlorotyrosine formation from free L-tyrosine (see Fig. 2 *A*, *left*) was performed in mixtures of phosphoric acid, monobasic and dibasic sodium phosphate (final concentration 50 mM). The pH of each reaction mixture was determined at the end of the incubation. For experiments involving L-tyrosine oxidation with reagent molecular chlorine, Cl_2 (Aldrich Chemical Company; Milwaukee, WI) was first purged into a vented gas-tight reservoir to one atmosphere of pressure, and a known quantity of Cl_2 was then removed and added to a sealed reaction vial utilizing a chloride-free gas-tight syringe rendered chlorine-demand-free (15).

Human neutrophils. Neutrophils were isolated by buoyant density centrifugation as previously described (22, 27). Hank's balanced salt solution (Mg^{2+}-, Ca^{2+}, phenol- and bicarbonate-free, pH 7.2) supplemented with 100 μM diethylenetriamine pentaacetic acid was used for neutrophil isolation. Neutrophil experiments were performed in Medium A (Mg^{2+}- and Ca^{2+}-containing Hank's balanced salt solution supplemented with 100 μM diethylenetriamine pentaacetic acid, pH 7.2).

To recover L-tyrosine oxidation products formed by neutrophils in the "frustrated phagolysosome," the supernatant was set aside, and the adherent cells were extracted with successive 2 ml washes with H_2O, methanol, H_2O, diethyl ether, and H_2O. Recovery of L-tyrosine was quantitative under these conditions. Preliminary experiments demonstrated the need for extraction with organic solvent presumably because amino acids were trapped beneath a membrane layer of the cells. 3-[$^{13}C_6$]Chlorotyrosine (20 pmol) and L-[$^{13}C_6$]tyrosine (100 nmol) internal standards were added to the combined extracts and supernatant, and the solution was concentrated to dryness under vacuum. Amino acids were then subjected to GC-MS analysis.

L-tyrosine was trapped within antibody sensitized sheep red blood cells (Sigma Chemical Co.) Erythrocyte ghosts containing L-tyrosine were prepared by creating reannealable pores in the membrane of the cells (28). All procedures were carried out at 4°C unless otherwise specified. Immunoglobulin sensitized sheep red blood cells pelleted by centrifugation (500 *g* for 5 min) were suspended in a hypotonic solution (5 mM sodium phosphate buffer, pH 7.4) containing L-tyrosine (2 mM) and incubated for 5 min. Cells were pelleted by centrifugation, resuspended in the same L-tyrosine-containing buffer, and incubated an additional 5 min. The cells were pelleted and reannealed by suspension in isotonic phosphate buffered saline supplemented with L-tyrosine (2 mM) and $MgCl_2$ (2 mM) at 37°C for 30 min. Cells were stored at 4°C and used the day of preparation.

To recover L-tyrosine oxidation products the leukocytes and red blood cells were pelleted by centrifugation (5,000 *g* for 10 min at 4°C) and the supernatant saved. The cell pellet was disrupted by addition of H_2O (1 ml) and incubated at 37°C for 10 min. Membranes were pelleted by centrifugation and the supernatant saved. After addition of [$^{13}C_6$]3-chlorotyrosine (20 pmol) internal standard, the combined supernatants were dried under vacuum and the 3-chlorotyrosine content in the residue determined by GC-MS analysis.

Reverse-phase HPLC quantitation of L-tyrosine oxidation products. L-tyrosine oxidation products were analyzed by reverse-phase high performance liquid chromatography (HPLC) with a C18 column (Beckman μPorasil, 5 μm resin, 4.6 × 250 mm) and quantified by comparison of integrated peak areas to standard curves generated using authentic 3-chlorotyrosine and L-tyrosine, respectively (27). The identities of the compounds generated by HOCl, Cl_2, myeloperoxidase and neutrophils were confirmed by gas chromatography-mass spectrometry.

[$^{13}C_6$]3-chlorotyrosine synthesis. NaOCl (final concentration 2 mM) was added dropwise to a constantly stirring solution of 2 mM L-[$^{13}C_6$]tyrosine in Buffer B (50 mM sodium phosphate, 100 mM NaCl, pH 2.0). TFA (0.1% final) was then added and 3-[$^{13}C_6$]chlorotyrosine isolated by reverse-phase HPLC. This procedure results in an approximate 50% yield of 3-[$^{13}C_6$]chlorotyrosine. Internal standard was stored at −20°C under argon.

Gas chromatography–mass spectrometry. Before derivatization, amino acids were resuspended in 1 ml Buffer A (0.1% TFA), immediately passed over a solid phase C18 extraction column (Supelclean LC-18 SPE tubes, 3 ml; Supelco Inc., Bellefonte, PA) equilibrated in Buffer A, washed with 2 ml Buffer A, and batch eluted with 50% methanol in Buffer A. The eluent was then dried under vacuum, and propyl esters of amino acids were prepared by the addition of 200 μl 3.5 M HBr in n-propanol (Cambridge Isotope Laboratories; Andover, MA) followed by heating at 65°C for 30 min. Propylated products were dried under N_2 and heptafluorobutyl derivatives were prepared by addition of 50 μl of 3:1 (vol/vol) ethyl acetate/hepta-fluorobutyric acid anhydride and heating at 65°C for 30 min (19, 22).

Derivatized products were concentrated to dryness under anhydrous N_2, reconstituted in heptane and analyzed on an 8 m capillary column (Hewlett-Packard Ultra performance, 0.17 μM dimethyl silicone film, i.d. 0.31 mm) by GC-MS (Hewlett Packard 5988) in the negative chemical ionization mode with methane (source pressure 1 Torr; source temperature 200°C) as the reagent gas. The injector and interface temperatures were both 280°C. The initial GC oven temperature was 85°C for 0.5 min followed by an increase of 20°C/min to 300°C. For experiments detecting Cl_2 in head space gas, samples were introduced into the mass spectrometer through a DB-1 column (25 m, 0.25 mm i.d., 0.32 μm film thickness; J & W Scientific) in the splitless injection mode. The column was run isothermally (25°C). Electron impact GC-MS analysis of p-hydroxyphenylacetaldehyde (pHA) was performed as previously described (27).

Other procedures. All procedures were carried out in chlorine-demand-free (15) and chloride-free glassware. Glassware was rendered chlorine-demand-free and chloride-free by soaking in chloride-free NaOCl, extensive rinsing with H_2O, and pyrolysis at 500°C overnight. Buffers were demonstrated to be chlorine-demand-free (defined as no detectable (< 3%) consumption of 1 mM HOCl in a 10-min incubation at 37°C within a gas-tight vial) as monitored by the oxidation of iodide to triiodide (29). H_2O_2 concentration was determined spectrophotometrically ($\epsilon_{240} = 39.4\ M^{-1}cm^{-1}$; reference 30).

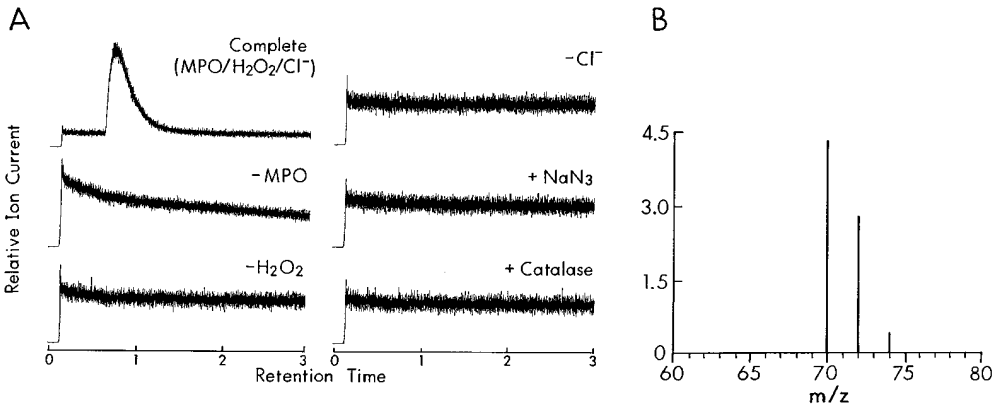


Figure 1. Mass spectrometric detection of myeloperoxidase-generated chlorine gas (Cl_2). (*A*) Myeloperoxidase (1.2 μM) was incubated at 37°C in the presence of H_2O_2 (four additions of 250 μM over 8 min via a gas-tight syringe) and NaCl (100 mM) in sodium phosphate buffer (50 mM; pH 4.5) in a sealed reaction vial (complete system). Dead space gas above the reaction mixture containing the complete myeloperoxidase-H_2O_2-Cl^- system, or the indicated additions and deletions, was then subjected to electron impact (70 eV) GC-MS analysis with monitoring of ions of m/z 70–74 as described in Methods. Where indicated, NaN_3 (1 mM) or catalase (20 μg/ml) was included. (*B*) Electron ionization mass spectrum of Cl_2 generated by the complete myeloperoxidase-H_2O_2-Cl^- system. Similar results were observed in three independent experiments.

Results

The myeloperoxidase-H_2O_2-Cl^- system generates Cl_2. Cl_2 is composed of two abundant isotopes of mass 35 and 37, and its mass spectrum demonstrates a characteristic isotopic cluster of ions at mass-to-charge ratios (m/z) of 70, 72, and 74. Electron impact GC-MS analysis of the gas phase above a reaction mixture containing the complete myeloperoxidase-H_2O_2-Cl^- system revealed ions with the expected m/z of Cl_2 (Fig. 1 *A*). The retention time of the gas, as well as its mass spectrum (Fig. 1 *B*), were indistinguishable from authentic Cl_2. Generation of Cl_2 required each component of the complete myeloperoxidase system; it was inhibited by the H_2O_2 scavenger catalase and the heme poison azide. These results indicate that Cl_2 is generated by the myeloperoxidase-H_2O_2-Cl^- system of phagocytes at acidic pH.

3-Chlorotyrosine is a Cl_2-specific oxidation product of free L-tyrosine. We were unable to demonstrate Cl_2 generation by activated human neutrophils as monitored by electron impact GC-MS analysis of head space gas above neutrophil incubations, probably due to the highly reactive nature of Cl_2. Indeed, current assays for HOCl generation do not detect HOCl directly, but rather monitor the oxidation of target molecules (31). We therefore sought to identify a Cl_2-specific oxidation product to use as an indicator of Cl_2 production by phagocytes. It has been suggested that 3-chlorotyrosine might serve as a specific marker for HOCl generation at sites of inflammation (8, 32). However, Cl_2 is a powerful electrophile that is widely utilized in the synthesis of halogenated aromatic compounds (33, 34), raising the possibility that Cl_2 mediates tyrosine chlorination by HOCl. Since Cl_2 is generated from HOCl only in the presence of Cl^- and H^+ (Eq. 1), we tested this hypothesis by determining the Cl^- dependence and pH optimum of 3-chlorotyrosine synthesis by reagent HOCl (Fig. 2). In the presence of plasma concentrations of Cl^-, chlorination of the aromatic ring of free L-tyrosine was optimal at acidic pH. In the absence of Cl^-, no 3-chlorotyrosine was observed, even under acidic conditions (Fig. 2 *A*). The acidic pH optimum and Cl^- dependence for the chlorination of the aromatic ring of L-tyrosine strongly suggest that Cl_2, and not HOCl itself, is the oxidizing intermediate in 3-chlorotyrosine synthesis.

To test further the role of Cl_2 in aromatic halogenation reactions, we investigated the synthesis of 3-chlorotyrosine from free L-tyrosine in hexane. In this aprotic, non-polar solvent,

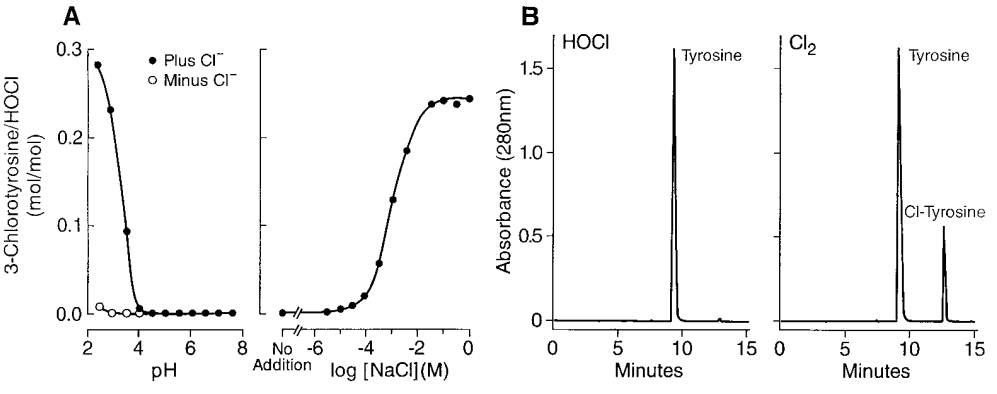


Figure 2. 3-chlorotyrosine is a Cl_2-specific oxidation product. (*A*) L-tyrosine (2 mM) was incubated for 30 min with chloride-free NaOCl (100 μM) within gas-tight vials at 37°C. 3-Chlorotyrosine produced was subsequently quantified by reverse-phase HPLC as described under Methods. 3-Chlorotyrosine identity was confirmed by GC-MS analysis. Values are the mean of duplicate determinations and are representative of the results observed in three independent experiments. (*A*, *left*) Reactions were carried out in 50 mM buffer (prepared using a mixture of sodium phosphate monobasic, sodium phosphate dibasic and phosphoric acid as described in Methods) in the absence or presence of NaCl (100 mM). The pH of the reaction mixture was determined at the end of the incubation. (*A*, *right*) Reactions were carried out at the indicated final concentration of NaCl in 50 mM sodium phosphate (pH 2.5). (*B*) L-Tyrosine (4 mg) in hexane (4 ml) was exposed to 10 nmol HOCl (extracted into hexane) or 10 nmol Cl_2 within sealed reaction vials. After a 5 min incubation at 37°C, products were washed with hexane, dissolved in H_2O and analyzed by reverse-phase HPLC. The chromatograms depicted are from a single representative experiment performed in duplicate on three independent occasions.

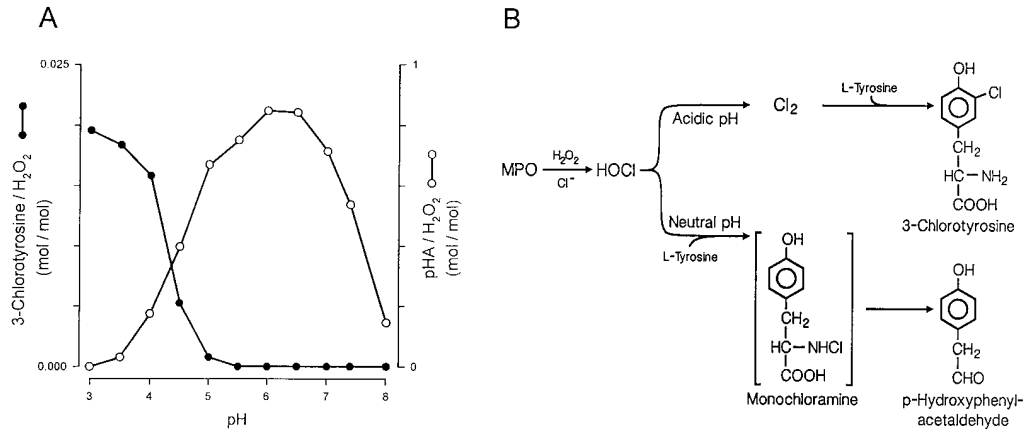
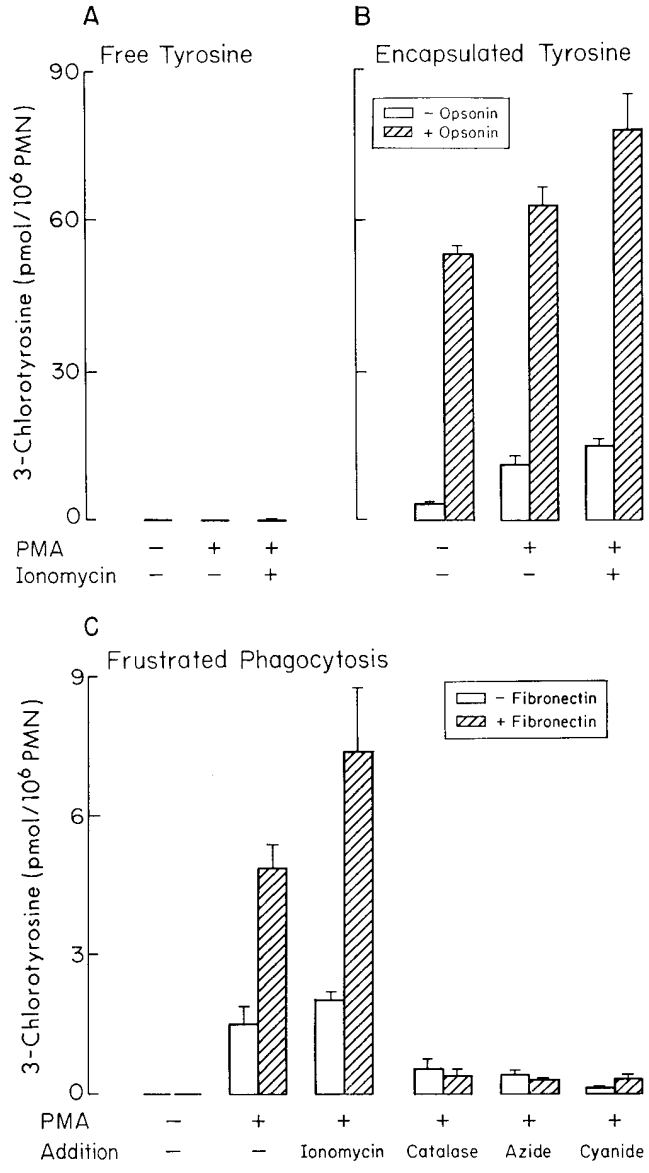


Figure 3. Myeloperoxidase oxidizes L-tyrosine by distinct chloride-dependent pathways at neutral and acidic pH. (*A*) L-tyrosine (100 μM), myeloperoxidase (40 nM), H_2O_2 (100 μM), and NaCl (100 mM) were incubated in sodium phosphate buffer (50 mM) at 37°C within sealed reaction vials for 30 min at the indicated pH. 3-Chlorotyrosine and p-hydroxy-phenylacetaldehyde (pHA) were then quantified by reverse-phase HPLC as described under "Methods." Identities of oxidation products were confirmed by GC-MS analysis. Values are the mean of duplicate determinations and are representative of the results observed in three independent experiments. (*B*) Proposed reaction pathways for L-tyrosine oxidation by the myeloperoxidase-H_2O_2-Cl^- system.



protons and halide are unavailable, and HOCl should not generate Cl_2. L-tyrosine was exposed to equimolar amounts of either Cl_2 or HOCl in sealed reaction vials, and the reaction products were then analyzed by reverse-phase HPLC. Only Cl_2 generated significant quantities of 3-chlorotyrosine (Fig. 2 *B*). In parallel studies, L-tyrosine was incubated in a chloride-free aqueous solution with equimolar quantities of either Cl_2 or HOCl; at both acidic and neutral pH, only Cl_2 resulted in 3-chlorotyrosine synthesis. Thus, 3-chlorotyrosine is a specific marker for Cl_2-mediated oxidation of free L-tyrosine via halogenation of the aromatic ring.

The predominant product of free L-tyrosine oxidation by the myeloperoxidase-H_2O_2-Cl^- system at neutral pH is p-hydroxyphenylacetaldehyde (pHA) (27). The mechanism may involve an unstable monochloramine intermediate that undergoes spontaneous deamination and decarboxylation (15, 27). To investigate the role of Cl_2 in pHA production, we determined the pH optimum and Cl^--dependence for aldehyde production by myeloperoxidase and HOCl. Synthesis of pHA by myeloper-

Figure 4. Human neutrophils generate 3-chlorotyrosine within the phagolysosome. Freshly prepared human neutrophils (1×10^6/ml) were incubated in Medium A supplemented with (*A*) free L-tyrosine (2 mM) or (*B*) L-tyrosine trapped within antibody sensitized sheep red blood cells (1×10^9/ml) in the presence of the indicated additions. Neutrophils were maintained in suspension by intermittent mixing for 60 min at 37°C, and 3-chlorotyrosine content was then determined by stable isotope dilution NCI GC-MS analysis of n-propyl ester, per heptafluorobutyl derivatized products as described under "Methods." Where indicated, phorbol ester (200 nM) and ionomycin (1 μM) were included. Red blood cells were opsonized with serum (37). (*C*) L-tyrosine (4 μmol in 2 ml H_2O) was dried onto the bottoms of sterile glass scintillation vials (2 cm diameter) under a stream of N_2. Where indicated, human fibronectin (10 μg), catalase (10 μg), NaCN (1 μmol) or NaN_3 (1 μmol) were also included. Neutrophils (1×10^6 in 1 ml) were then added to the vials, permitted to adhere to the L-tyrosine-coated surface by incubation at 37°C for 30 min, and then stimulated by addition of phorbol ester (200 nM) and ionomycin (1 μM) as indicated. After an additional 30 min incubation at 37°C, 3-chlorotyrosine production was determined by stable isotope dilution NCI GC-MS. Values represent the mean±SEM of three determinations and are representative of results observed in three independent experiments.

oxidase is nearly quantitative at neutral pH and physiological Cl^- concentrations (27). However, at acidic pH and physiological Cl^- concentrations, 3-chlorotyrosine was produced (Fig. 3 *A*), suggesting that different oxidants were mediating chlorination of the aromatic and amine moieties of L-tyrosine. In contrast to 3-chlorotyrosine (Fig. 2), pHA was generated from L-tyrosine by HOCl in aprotic solvents and in Cl^--free aqueous solutions (data not shown). Collectively, these results indicate that 3-chlorotyrosine and pHA constitute distinct oxidation products generated by the myeloperoxidase-H_2O_2-Cl^- system; Cl_2-mediates electrophilic addition of the aromatic ring, and HOCl-mediates chlorination of the α-amino moiety (Fig. 3, *right*). 3-Chlorotyrosine synthesis from free L-tyrosine thus serves as a specific marker of Cl_2 generation, and it would be anticipated to occur most readily within an acidic micro-environment like the phagolysosome (35, 36), where myeloperoxidase and H_2O_2 are secreted (3, 9).

Human neutrophils generate 3-chlorotyrosine, a Cl_2-specific oxidation product of free L-tyrosine, during phagocytosis. Human neutrophils rapidly phagocytose complement- and immunoglobulin-coated sheep red blood cells (9, 37–40). To determine whether Cl_2 might be generated in the phagolysosome, we encapsulated L-tyrosine within immunoglobulin sensitized sheep red blood cells, incubated the cells with human neutrophils, and then monitored 3-chlorotyrosine synthesis. When sheep red blood cells were coated with both immunoglobulin and complement (37, 38), substantial quantities of 3-chlorotyrosine were generated (Fig. 4 *B*). Microscopic examination of reaction mixtures revealed intracytoplasmic inclusions within the neutrophils, confirming erythrophagocytosis under these conditions. Further stimulation with phorbol ester or phorbol ester and calcium ionophore resulted in small but significant increases ($P < 0.05$ and $P < 0.01$, respectively; ANOVA) in L-tyrosine oxidation (Fig. 4 *B*). When red blood cells were coated with immunoglobulin alone, incubation with neutrophils produced smaller quantities of 3-chlorotyrosine, consistent with the well-established role of complement in promoting phagocytosis (9, 37, 38). Although opsonized and immunoglobulin-coated sheep red blood cells without encapsulated L-tyrosine were avidly phagocytosed by neutrophils, free 3-chlorotyrosine production was undetectable. Moreover, neutrophils exposed to free L-tyrosine failed to generate significant levels of 3-chlorotyrosine, even when stimulated with both phorbol ester and calcium ionophore (Fig. 4 *A*). Instead, the predominant L-tyrosine oxidation product was pHA (38 ± 6 nmol/10^6 PMN; mean±SEM, $n = 3$). Thus, human neutrophils generated significant levels of the Cl_2-specific oxidation product, 3-chlorotyrosine, only when L-tyrosine was delivered to the phagolysosome.

White blood cells plated on a glass surface coated with phagocytosis-promoting ligands generate a closed compartment, termed the "frustrated phagolysosome," where advancing pseudopodia form a tight seal with the underlying matrix (41, 42). As an independent test of the role of phagocytosis in Cl_2 generation, neutrophils were applied to L-tyrosine-coated glass vials, and 3-chlorotyrosine production was determined. In the absence of phorbol ester, no significant 3-chlorotyrosine was generated, suggesting that neutrophil adherence to a tyrosine-coated surface fails to trigger activation of the NADPH oxidase and secretion of myeloperoxidase into the frustrated phagolysosome. This contrasts markedly with immunoglobulin coated sheep red blood cells, which are known to activate $O_2^{\cdot -}$ production and myeloperoxidase secretion (Fig. 4 B and reference 37). However, adherent neutrophils generated significant levels of 3-chlorotyrosine when stimulated with either phorbol ester or phorbol ester and calcium ionophore in the presence of fibronectin (Fig. 4 *C*). This basement membrane protein facilitates leukocyte adhesion, promotes phagocytosis, and primes neutrophils for oxidant generation (43, 44). Even in the absence of fibronectin, cells activated with phorbol ester generated significant levels of 3-chlorotyrosine, consistent with enhanced $O_2^{\cdot -}$ production, myeloperoxidase secretion and/or increased neutrophil adherence. Addition of the peroxidase inhibitors N_3^- and CN^- blocked 3-chlorotyrosine production. The peroxidase scavenger catalase, when free in solution, failed to inhibit 3-chlorotyrosine synthesis. However, catalase applied to the L-tyrosine-coated surface markedly attenuated 3-chlorotyrosine formation (Fig. 4 *C*), consistent with the observation that the frustrated phagolysosome is inaccessible to high molecular weight proteins free in solution (41). Thus, in two independent models of phagocytosis, human neutrophils converted L-tyrosine to 3-chlorotyrosine, indicating that a halogenating agent with characteristics identical to that of Cl_2 serves as a physiological oxidant within the phagolysosome.

Discussion

Several lines of evidence indicate that human neutrophils employ myeloperoxidase to generate a Cl_2-like oxidant: (*a*) Electron impact GC-MS analysis of head space gas above reaction mixtures containing the complete myeloperoxidase-H_2O_2-Cl^- system demonstrated Cl_2 production. (*b*) Both Cl^- and H^+ were required for the conversion of free L-tyrosine into 3-chlorotyrosine by reagent HOCl, strongly suggesting that the reaction involved the equilibrium-driven formation of Cl_2. (*c*) Cl_2, but not an equimolar amount of HOCl, mediated halogenation of the aromatic ring of L-tyrosine in the absence of Cl^- at neutral pH. This observation, together with the Cl^- and H^+ dependence of chlorination by HOCl, indicate that 3-chlorotyrosine is a specific marker for Cl_2-mediated oxidation of free L-tyrosine. (iv) In two independent models of phagocytosis human neutrophils converted free L-tyrosine to 3-chlorotyrosine. In both systems, 3-chlorotyrosine synthesis was inhibited by heme poisons and catalase, implicating myeloperoxidase and H_2O_2 in the reaction. Collectively, these results indicate that the myeloperoxidase H_2O_2-Cl^- system generates Cl_2 under acidic conditions, that Cl_2 mediates the synthesis of 3-chlorotyrosine, and that human neutrophils employ a halogenating agent with characteristics identical to that of Cl_2 as an oxidant within the phagolysosome.

It is noteworthy that the milieu of the phagolysosome is precisely orchestrated to provide optimal oxidative and proteolytic conditions for destruction of invading pathogens (9). Our results indicate that this environment also promotes the production of Cl_2, a potent electrophilic reagent. Cl_2 produced within neutrophil phagolysosomes may be derived from myeloperoxidase-generated HOCl via the equilibrium described in Eq. 1. Another pathway for Cl_2 generation may involve chloramines or other chlorinated intermediates derived from HOCl:

$$R_2NC1 + C1^- + H^+ = R_2NH + C1_2 \quad (2)$$

Indeed, chloramines are utilized synthetically (33, 34) to halogenate aromatic groups in a Cl^- and H^+ dependent reaction through generation of Cl_2 (Eq. 2). The chlorination of protein-bound tyrosine, however, may involve additional mechanisms. Domigan et al. (reference 32) recently suggested that HOCl-mediated chlorination of tyrosine within a peptide was sequence-specific and proposed an intramolecular mechanism involving a monochloramine intermediate. However, these conclusions were based on indirect evidence and the underlying mechanism(s) for protein-bound tyrosine chlorination remains to be determined.

The pH dependence of 3-chlorotyrosine formation from free L-tyrosine in vitro suggests that formation of this Cl_2-generated molecule by myeloperoxidase may occur in acidic compartments in vivo. The plasma membrane of adherent phagocytes generates a closed compartment where advancing pseudopodia form a tight seal with the underlying matrix (41). Direct examination of these microenvironments reveals a $pH < 4$ (45).

Molecular chlorine generated by myeloperoxidase may also play an important role in atherosclerosis because hypoxic conditions, resulting from impaired oxygen diffusion, are likely to render the artery wall acidic (46). Oxidation-specific epitopes are present in lysosomal-like structures in the macrophages of atherosclerotic tissue (47), and phagocytosis is a potent stimulus for the secretion of myeloperoxidase and H_2O_2 into the phagolysosome (3, 9, 14). This compartment is optimized for the oxidative damage of proteins and lipids, and provides the acidic environment (35, 36) that is ideal for Cl_2 generation by myeloperoxidase. These observations suggest that Cl_2 generated by myeloperoxidase in the artery wall (48) may play a role in oxidizing low density lipoprotein, which in turn may be of central importance in triggering the pathological events of atherosclerosis (8, 10). Indeed, monoclonal antibodies specific for $HOCl/ClO^-$-modified proteins recognize epitopes in atherosclerotic lesions (49), and we have recently demonstrated that low density lipoprotein cholesterol is a major target for oxidation by the myeloperoxidase-H_2O_2-Cl^- system of phagocytes at acidic pH, yielding a family of chlorinated sterols (50). Furthermore, Cl_2 derived from HOCl, and not HOCl itself, appears to be the reactive intermediate in cholesterol chlorination (50).

Our results suggest that Cl_2 should be added to the growing list of gases that play important physiological roles. The selective biological reactivity of Cl_2, coupled with the generation of 3-chlorotyrosine by human neutrophils, suggests that Cl_2 may play an important role in host defenses and inflammation. Detection of Cl_2-dependent oxidation products in vivo would strongly implicate Cl_2-mediated halogenation as a physiologically significant oxidation pathway employed by phagocytes at sites of inflammation and vascular disease.

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