Reactions of Hypochlorous Acid with Tyrosine and Peptidyl-tyrosyl Residues Give Dichlorinated and Aldehydic Products in Addition to 3-Chlorotyrosine*

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The toxicity of hypochlorous acid (HOCI) generated from activated neutrophils has been associated with several pathological processes such as atherosclerosis. Formation of 3-chlorotyrosine (Cl-Tyr) has been used as a marker for assessing the involvement of HOCI in such processes. In this study, we aimed to investigate the formation of Cl-Tyr from reaction of HOCI with tyrosine (both free and peptide-bound) and the fate of Cl-Tyr under such conditions. Tyrosine, N-acetyltyrosine, bovine serum albumin, and human low density lipoproteins were incubated with a range of reagent hypochlorite concentrations for varying periods in 10 mM phosphate buffer (pH 7.4) at 22 °C. The reaction products, and several biological samples, were hydrolyzed (in the case of proteins), isolated, and purified by high pressure liquid chromatography and characterized or quantitated by mass spectrometry and NMR. A significant amount of 3,5-dichlorotyrosine (diCl-Tyr) was obtained from the bovine serum albumin, low density lipoprotein, and some biological samples, in addition to Cl-Tyr, indicating that Cl-Tyr competes effectively for HOCI even when tyrosine is present in great excess. Cl-Tyr and diCl-Tyr were also formed from free tyrosine but then reacted further with HOCI. This finding differs from a claim in the literature that Cl-Tyr was not formed in such a system. The further reaction products of Cl-Tyr and diCl-Tyr with HOCI were elucidated as their corresponding mono- and dichlorinated 4-hydroxyphenylacetaldehydes. These results indicate the importance of assessing other products of HOCI action in addition to Cl-Tyr.

Stimulated neutrophils generate reactive oxygen species, such as superoxide anion radicals (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)), and can secrete the enzyme myeloperoxidase (1). Reaction of myeloperoxidase with H\(_2\)O\(_2\) in the presence of physiological concentrations of chloride (Cl\(^-\)) results in the formation of the powerful oxidant hypochlorous acid (HOCI)\(^1\) (2).

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\(^1\) The abbreviations used are: HOCI, hypochlorous acid; LDL, low density lipoprotein; Cl-Tyr, 3-chlorotyrosine; diCl-Tyr, 3,5-dichlorotyrosine; Ac-dCl-Tyr, N-acetyl-3,5-dichlorotyrosine; Ac-Tyr, N-acetyltirosine; Ac-Cl-Tyr, N-acetyl-3-chlorotyrosine; HPAA, 4-hydroxyphenylacetaldehyde; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ODS, octadecylsilanized silica; SES-MS, electrospray-mass spectrometry; Cl-HPAA, 3-chloro-4-hydroxyphenylacetaldehyde; diCl-HPAA, 3,5-dichloro-4-hydroxyphenylacetaldehyde; HPLC, high pressure liquid chromatography.

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terminus or on a lysine side chain) or chloramidine group on a peptide bond adjacent to a tyrosyl residue in a peptide or a protein undergoes an intramolecular reaction with the tyrosyl residue to convert it into CI-Tyr (47).

In this study, we aimed to investigate both the formation of CI-Tyr from reaction of HOCl with tyrosine (both free and peptide-bound) at pH 7.4 and the fate of CI-Tyr under such conditions. We have found that CI-Tyr is not only formed from tyrosine (both free and peptide-bound) but also reacts further with excess HOCl to give rise to other oxidation products such as 3,5-dichlorotyrosine (diCl-Tyr) and aldehydes such as chlorinated HPAA. Accordingly, we have detected diCl-Tyr on proteins obtained from in vivo inflammatory sites characterized by neutrophil infiltration.

MATERIALS AND METHODS

The water used was filtered through a four-stage Milli Q system (Millipore-Waters, Lane Cove, New South Wales, Australia) equipped with a 0.2-μm pore size final filter. Tyrosine, N-acetyltyrosine (Ac-Tyr), CI-Tyr standard, and bovine serum albumin (BSA, fatty acid-free) were from Sigma. Isotopically enriched phosphate-buffered saline (PBS, 50 mM NaH2PO4, pH 7.4) was prepared in nanopure water and treated overnight with Chelex 100 resin (Bio-Rad) to remove contaminating metal ions (52). All other chemicals, solvents, and chromatographic materials were of analytical or HPLC grade.

Preparation of HOCl Reagent—HOCl solutions (10 mM) were prepared daily by dilution of a concentrated stock solution (approximately 1 M in 0.1 M sodium hydroxide, BDH, Poole, Dorset, UK) into 10 mM phosphate buffer (pH 7.4). The concentration of HOCl was determined by measuring its absorbance at 290 nm at pH 9.5, based on a molar absorptivity of 350 dm3 mol⁻¹ cm⁻¹ for the OCI species.

Preparation of LDL from Human Plasma—Human LDL (density 1.019-1.063) was prepared by density gradient ultracentrifugation of plasma from normal fasting donors (53). Protein content of LDL was determined using bicinchoninic acid method (Sigma), with BSA as standard.

Preparation of Biological Samples for Analysis of Chlorinated Tyrosines—Samples of normal human iliac vessels and advanced carotid atherosclerotic plaques (54), of normal and stage IV cutaneous human lenses (55), and of peritoneal abscesses from the mouse, were extracted to prepare materials ready for protein preparation. Mice were injected with 2×10⁶ colony-forming units/ml E. coli, 10⁹ colony-forming units/ml Bacteroides fragilis, 10⁹ colony-forming units/ml Enterobacter cloacae, 10⁹ colony-forming units/ml Acinetobacter baumannii, 10⁷ colony-forming units/ml B. subtilis, 10³ colony-forming units/ml B. melinonii, 10⁹ colony-forming units/ml L. casei, 5×10⁸ colony-forming units/ml Lactobacillus acidophilus, and 5×10⁷ colony-forming units/ml Lactobacillus plantarum, intraperitoneal, as described in detail previously (56). Abscesses were removed 7 days later and frozen in liquid nitrogen for brief storage at −70 °C. These samples were kindly provided to us by Dr. John Finlay-Jones, Flinders University, Adelaide, Australia.

The abscesses frozen in liquid nitrogen were powdered in an Epennodentoe tube by using a Teflon hand homogenizer. Proteins were extracted from the abscess materials due to influence of background materials in the liquid nitrogen. For isolation of the products from the reaction of Ac-Tyr with HOCl, a semi-preparative ODS column (LC-18, 25×1 cm, 5-μm particle size, Supelco) was used. Separation of the products was achieved by isocratic elution with 6% methanol and 0.06% triethylammonium acetate (v/v) in water at a flow rate of 3 mL/min. Sample collection was automated by the use of an FPLC-10A fraction collector (Shimadzu). Collection windows were determined based on the UV absorbance of the eluent at 280 nm.

DiCl-Tyr present in biological samples was determined on a Shimadzu LC-10Avp HPLC system equipped with a CouArray electrochemical detector (model 5600, ESA, Chelmsford, MA) with 12 cells (channels) in series using platinum electrodes. The chromatographic conditions were the same as described above for the detection of CI-Tyr, except for the gradient elution profile that was modified as follows: isocratic elution with 6% solvent B for 16 min; then to 8% solvent B for 4 min; further elution at 8% solvent B for 10 min; then to 80% solvent B for 5 min before re-equilibration with 6% solvent B for 15 min. The potential settings for the CouArray detector were 150, 310, 380, 450, 500, 530, 550, 560, 620, 700, 710, and 840 mV, for channels 1-12 respectively. The signals for diCl-Tyr appeared in channels 6–8, with channel 7 as the dominant channel (where a maximum signal response was observed), channel 6 the leading channel and channel 8 the following one. Peak identity for diCl-Tyr in biological samples was confirmed by comparison of its hydrodynamic voltammogram and the two “ratio chromatograms” (the two “ratio chromatograms” were determined using both diode array and electrochemical detection) by comparison of its hydrodynamic voltammogram and the two “ratio chromatograms” (the two “ratio chromatograms” were determined using both diode array and electrochemical detection) with that of standard diCl-Tyr (58). To add to further identification, standard diCl-Tyr was also spiked into the biological samples and analyzed subsequently for the fractional increment of the added substance. The detection limit of diCl-Tyr by this method was determined to be 4 μmol/mol Tyr for standard and 40 μmol/mol Tyr for the biological samples due to influence of background materials in the samples.

MS and NMR—Electrospray (ES) MS was conducted on a VG Platform mass spectrometer (Fisons, New South Wales, Australia). The samples (around 100 μmol/ml) were dissolved in 50% aqueous acetonitrile. The solvent was delivered by a Phoenix (Fison) syringe pump at a flow rate of 20 μl/min; 10 μl of each solution was injected for analysis. A dry nitrogen bath gas at atmospheric pressure was employed to assist evaporation of the electrospray droplets. A negative electrospray mode was used with capillary potential at 2.35 kV, chicane counter electrode potential at 0.5 kV, and cone potential at 45 V.

Proton NMR studies were performed on a Varian Gemini 300 BB spectrometer at 22 °C. Samples were dissolved in 0.01 M HCl in H2O. The chemical shifts were referenced to the non- or partially deuterated water signal that was set to 4.76 ppm. Ac-Tyr, δ ppm 1.84 s (3H, Ac-CH3), 2.83 dd (8.6 Hz, 14.1 Hz, 1H, β-CH2), 3.02 dd (5.6 Hz, 14.1 Hz, 1H, β-CH2), 4.46 dd (5.6 Hz, 8.6 Hz, 1H, α-CH), 6.74 (8.5 Hz, 2H, 2-H, 6-H), 7.04 dd (8.5 Hz, 2H, 3-H, 5-H).

Ac-Tyr: δ ppm 1.82 s (3H, Ac-CH3), 2.71 dd (8.7 Hz, 14.0 Hz, 1H, β-CH2), 2.98 dd (5.0 Hz, 14.0 Hz, 1H, α-CH), 5.01 d (5.0 Hz, 1H, 6-H), 6.97 dd (2.5 Hz, 8.5 Hz, 1H, 6-H), 7.16 (2.3 Hz, 1H, 2-H). Ac-diCl-Tyr: δ ppm 1.84 s (3H, Ac-CH3), 2.78 dd (8.9 Hz, 14.1 Hz, 1H, β-CH2), 3.03 dd (5.0 Hz, 14.1 Hz, 1H, β-CH2), 4.50 dd (5.0 Hz, 8.9 Hz, 1H, α-CH), 7.12 s (2H, 2-H, 6-H). CI-Tyr: δ ppm 2.99 dd (7.7 Hz, 14.8 Hz, 1H, β-CH2), 3.11 dd (7.5 Hz, 14.8 Hz, 1H, β-CH2), 4.03 dd (5.5 Hz, 7.7 Hz, 1H, α-CH), 6.90 (8.8 Hz, 1H, 5-H). diCl-Tyr: δ ppm 2.97 dd (7.7 Hz, 14.7 Hz, 1H, β-CH2), 3.09 dd (5.5 Hz, 7.7 Hz, 1H, α-CH).
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RESULTS

Preparation and Structural Identification of diCl-Tyr from Reaction of HOCl with Ac-Tyr—Ac-Tyr (2 mM) in 10 mM phosphate buffer (pH 7.4) was reacted with HOCl at 22 °C. The reaction mixture was immediately chromatographed on a Zorbax ODS column and monitored at 280 nm. Apart from the unreacted parent Ac-Tyr that elutes at 7.5 min, there were two distinct products eluting at 14.7 and 20.4 min, respectively, in the chromatogram (Fig. 1). Product formation was essentially instantaneous, and prolonged reaction time did not increase the yields of the two products. However, addition of further aliquots of HOCl to the reaction mixture resulted in an enhanced loss of Ac-Tyr and an increase in the size of the two product peaks. When HOCl was in large excess to the original Ac-Tyr added (e.g. 2:1), the peak at 20.4 min became the predominant one, concomitant to a loss of the compound eluting at 14.7 min, and a further loss of Ac-Tyr (HPLC trace not shown). These results indicate that the product eluting at 20.4 min in the HPLC is derived from the one eluting at 14.7 min. Fractions corresponding to these two products were collected from large scale reactions using 5 mM Ac-Tyr and HOCl separated on a semi-preparative ODS column (see under “Material and Methods”). The two fractions pooled from 20 separate injections (50 µl each injection) were then lyophilized before electrospray MS and proton NMR studies.

On the basis of ES-MS (Table I), 1H NMR (see “Materials and Methods”) and chemical reactions, the two oxidation products of Ac-Tyr eluting at 14.7 and 20.4 min as shown in Fig. 1 are assigned to N-acetyl-3-chlorotyrosine (Ac-Cl-Tyr, I) and N-acetyl-3,5-dichlorotyrosine (Ac-diCl-Tyr, II), respectively, as shown in Scheme 1.

Ac-Cl-Tyr gives two M − 1 ions in ES-MS (in negative ion mode) at m/z 256/258 with a ratio of 3:1 (Table I), suggesting the product contains one Cl atom on the basis that the natural abundance of the two Cl isotopes, 35Cl and 37Cl, is 3:1. 1H NMR data support the occurrence of a mono-chlorinated aromatic ring, as evidenced by the asymmetrical appearance of the signals for 3 protons in the aromatic region of the product when compared with the symmetrical signals for 4 aromatic protons in Ac-Tyr. On acid hydrolysis (catalyzed by 6 M HCl), a product identical to authentic Cl-Tyr was obtained as assessed by HPLC (chromatogram not shown) and spectroscopy (ES-MS, see Table I, and 1H NMR studies, see under “Materials and Methods”). Thus this product is confirmed as Ac-Cl-Tyr.

The evidence of two Cl atoms in Ac-diCl-Tyr was also derived from ES-MS and NMR studies. The distribution of the M − 1 ion cluster at m/z 290/292/294 (9:6:1) is characteristic of a dichlorinated compound. A singlet signal at δ 7.12 ppm attributable to two protons in the aromatic region of the 1H NMR spectrum indicates that the two protons are chemically and electromagnetically equivalent. Thus the two Cl atoms could be either at positions 3 and 5 or at positions 2 and 6. Since Ac-diCl-Tyr is most likely derived from further reaction of Ac-Cl-Tyr with HOCl, and since 2-chlorotyrosine was not observed in the reaction mixture, the two Cl atoms in Ac-diCl-Tyr have to be at positions 3 and 5. On acid hydrolysis (6 M HCl), Ac-diCl-Tyr yields diCl-Tyr which was characterized by both ES-MS (Table I) and NMR studies (see under “Materials and Methods”). These data confirm the structural identity of Ac-diCl-Tyr.

Since diCl-Tyr is not commercially available, its purity from our preparation was determined by NMR. 1H NMR spectra were obtained after addition of a known quantity of diCl-Tyr solid (0.54 mg) into 0.7 ml of 3.1 mM Cl-Tyr (Sigma) in D2O. Based on the peak areas of the signals of the aromatic protons, a molar ratio of the two compounds in the mixture was calculated, and the absolute quantity of diCl-Tyr in the solution was derived. The purity of our diCl-Tyr standard was determined to be 96% according to this method.

Reaction of HOCl with Free Tyrosine—Tyrosine (2 mM) in 10 mM phosphate buffer (pH 7.4) was reacted with HOCl (1, 0.2, or 0.1 mM) at 22 °C. The reaction mixture was immediately chromatographed on a Zorbax ODS column with both UV (at 280 nm) and EC (at 650 nm) detections. A typical HPLC of the reaction mixture at an early reaction time point is shown in Fig. 2. The two peaks at 24.5 and 31.4 min are due to Cl-Tyr and diCl-Tyr, respectively, based on comparison of their elution position and UV spectra with standards. The large peak at 27.6 min is attributed to HPAA by ES-MS analysis of an isolated and purified fraction (Table I). The HPAA fraction collected from the HPLC was freeze-dried and the residue re-dissolved in equal volumes of ethyl acetate and water. After separation, the ethyl acetate portion was evaporated and the remaining material examined using ES-MS (in negative ion mode). The major ion was found at m/z 135 (M−1), which is correct for HPAA (molecular weight 136). Full structural studies on HPAA formed from reaction of tyrosine with HOCl have been previously published by others (46). Product formation from reaction of free tyrosine with HOCl was slower than that observed with Ac-Tyr. When 2 mM Tyr was reacted with 1 mM HOCl, immediate HPLC analysis of the reaction mixture (approximately after 2 min reaction) showed that both Cl-Tyr and diCl-Tyr were formed at concentrations of 69.7 and 4.25 µM, respectively (Fig. 3). These two species decreased in concentrations as the reaction proceeded. By 3.5 h, they reached levels of 30.0 and 0.76 µM, respectively, in the reaction mixture. Over this time, the concentration of the parent amino acid tyrosine decreased from its initial value of 2.0 mM to 1.82 mM at 2 min, and 1.12 mM at 3.5 h. HPAA, on the other hand, increased its peak area by approximately 30-fold between 2 min and 3.5 h. Similar reaction kinetics were observed on reaction of 2 mM Tyr with both 0.2 mM HOCl (Fig. 3) and 0.1 mM HOCl. When 0.1 mM HOCl was used (where the molar ratio of tyrosine to HOCl reacted reached 20), the concentrations of Cl-Tyr, diCl-Tyr, and tyrosine were 4.50 µM, 0.43 µM, and 1.97 mM respectively, after 2 min reaction, and 4.05 µM, 0.09 µM, and 1.93 mM accordingly after a further 30-min reaction.
TABLE I

| Compound          | M – 1 ion (% intensity) | Compound          | M – 1 ion (% intensity) | Compound          | M – 1 ion (% intensity) |
|-------------------|-------------------------|-------------------|-------------------------|-------------------|-------------------------|
| Ac-Tyr            | 222 (100)               | Tyr               | 180 (100)               | HPAA              | 135 (100)               |
| Ac-Cl-Tyr         | 256 (100)               | Cl-Tyr            | 214 (100)               | Cl-HPAA           | 169 (100)               |
| Ac-diCl-Tyr       | 258 (33)                | diCl-Tyr          | 216 (33)                | diCl-HPAA         | 171 (33)                |
|                   | 290 (100)               | diCl-HPAA         | 248 (100)               |                   |                         |
|                   | 292 (67)                |                   | 250 (67)                |                   |                         |
|                   | 294 (11)                |                   | 252 (11)                |                   |                         |

The peak areas for HPAA were increased by approximately 8-fold between 2 and 30 min reaction.

Collectively, these data indicate that reaction of tyrosine with HOCl results in formation of HPAA, Cl-Tyr, and diCl-Tyr. Both Cl-Tyr and diCl-Tyr react further with HOCl to give rise to other oxidation products. In order to identify these further products, a new HPLC method that facilitates elution of less polar materials was developed. The method involves a gradient elution at 1 ml/min of solvent A, 5% MeOH in 0.1% trifluoroacetic acid in water, and solvent B, 80% MeOH in water on the same Zorbax ODS column. A representative HPLC of the reaction mixture of tyrosine (2 mM) and HOCl (1 mM) after 5 h reaction is shown in Fig. 4a. Under these conditions, tyrosine elutes at 6.7 min and HPAA at 10.8 min. Concomitant with the disappearance of both Cl-Tyr and diCl-Tyr were the appearance of several extra peaks at longer elution times. Two fractions corresponding to the major peaks at 17.1 and 21.4 min were collected and lyophilized. After partition of the fractions between ethyl acetate and water, the organic extracts were analyzed by ES-MS (in negative ion mode). The data summarized in Table I are consistent with an assignment of these two compounds as 3-chloro-4-hydroxyphenylacetaldehyde (Cl-HPAA) and 3,5-dichloro-4-hydroxyphenylacetaldehyde (diCl-HPAA), respectively.

Another possible precursor for Cl-HPAA and diCl-HPAA is the HPAA from reaction of HOCl with tyrosine. Isolated and purified HPAA (approximately 1 mM) (see Fig. 4b) was reacted with HOCl (1 mM) for 10 min at 22 °C, and the reaction mixture was subsequently analyzed by HPLC (Fig. 4c). Both Cl-HPAA and diCl-HPAA were formed when HOCl was reacted with HPAA. These results demonstrate that HOCl can chlorinate the aromatic ring of HPAA and suggest that HPAA can also act as a precursor of Cl-HPAA and diCl-HPAA.

**Reaction of HOCl with Human LDL—**It has been previously reported that exposure of LDL to HOCl resulted in immediate and preferential oxidation of amino acid residues of apolipoprotein B-100, the single protein associated with LDL. Neither lipoprotein lipid nor LDL-associated antioxidants represent major initial targets for this oxidant. Lysine residues of apolipoprotein B-100, the single protein associated with LDL. Neither lipoprotein lipid nor LDL-associated antioxidants represent major initial targets for this oxidant. Lysine residues of apolipoprotein B-100 were found to represent quantitatively the major target, scavenging about 70% of the HOCl added (10, 11). Reaction of HOCl with LDL is shown in Fig. 5. Both Cl-Tyr and diCl-Tyr were detected at all molar ratios investigated, with the yields proportional to the amount of HOCl present. For example, with a 5-fold molar excess HOCl to BSA (30 μM), the amounts of Cl-Tyr and diCl-Tyr formed were at 3.7 and 0.8 μM, respectively. With a 25-fold excess, the concentrations of these two products were at 37 and 6.8 μM.

**Reaction of HOCl with Human LDL—**It has been previously reported that exposure of LDL to HOCl resulted in immediate and preferential oxidation of amino acid residues of apolipoprotein B-100, the single protein associated with LDL. Neither lipoprotein lipid nor LDL-associated antioxidants represent major initial targets for this oxidant. Lysine residues of apolipoprotein B-100 were found to represent quantitatively the major target, scavenging about 70% of the HOCl added (10, 11). In this study, freshly isolated human LDL (3 μM) in PBS (50 mM, pH 7.4) was incubated with HOCl (1.2 mM) on ice for 15 min. The proteins in LDL (apolipoprotein B-100) were extracted and hydrolyzed. The amount of Cl-Tyr and diCl-Tyr was found to be 52.2 and 4.1 μM, respectively, in the hydrolysate (Fig. 6).
Chlorinated Tyrosines in in Vivo Samples—Four separate abscesses from WT/BL mice were analyzed for protein-bound Cl-Tyr and diCl-Tyr as described under “Materials and Methods.” Cl-Tyr was present at 804.1 $\pm$ 177.8 mmol/mol Tyr (mean $\pm$ S.D.) and diCl-Tyr at 77.5 $\pm$ 24.5 mmol/mol Tyr; thus generally diCl-Tyr was present at approximately 10% of the concentration of Cl-Tyr, in accordance with the in vitro results described above. Five other abscess samples from BALB/c and BL/4GKO IL-4 knockout mice were also analyzed; all were positive for Cl-Tyr, one was positive for diCl-Tyr, and the remainder were below our detection limit. This confirms that under these pathological conditions a high flux of chlorinating species, presumably from the large numbers of infiltrating neutrophils known to be present (56), can both mono- and dichlorinate proteins. In contrast, diseased human lenses (almost devoid of neutrophils) and advanced atherosclerotic plaques (characterized rather by mononuclear phagocytes than neutrophilic leukocytes) did not yield detectable levels of diCl-Tyr.

**DISCUSSION**

It has been shown previously that Cl-Tyr is formed from tyrosyl residues in peptides and proteins on exposure to HOCl (47, 49). However, the data presented here show that the formed Cl-Tyr undergoes further structural modifications in such systems. Thus when Ac-Tyr is treated with HOCl, both Ac-Cl-Tyr and Ac-diCl-Tyr are formed. Kinetic studies reveal that the mono-chlorinated tyrosine species reacts further with HOCl to give rise to the dichlorinated species. This conclusion is supported by studies on the reaction of HOCl with BSA and apolipoprotein B-100 in LDL. Thus when BSA (30 mM) was reacted with HOCl at varying concentrations, formation of both Cl-Tyr and diCl-Tyr was observed (see Fig. 5), although the yield of these two materials does not account for all the tyrosine lost nor HOCl added. For example, at 750 mM HOCl, 37 mM Cl-Tyr and 6.8 mM diCl-Tyr were formed on BSA, whereas 58 mM tyrosine was lost. The mono- and dichlorotyrosines therefore account for approximately 76% of the tyrosine lost and approximately 7% of the total HOCl added to the BSA solution. These results are very similar to those with BSA and in agreement with literature reports (10, 11). They have also confirmed that lipid components in LDL do not compete effectively for HOCl and therefore do not alter the reaction kinetics.
of HOCl with proteins. Our results with BSA and LDL indicate that approximately 10% of the oxidant is consumed in reactions with tyrosyl residues.

Free tyrosine, on the other hand, gives rise to a more complex mixture of oxidation products at physiological conditions on exposure to HOCl. Among the isolated and purified products are Cl-Tyr, diCl-Tyr, HPAA, Cl-HPAA, and diCl-HPAA (Figs. 3 and 4a). On the basis of the experimental results obtained in this study, we propose that HPAA and Cl-Tyr are the initial products, which both react further with HOCl and lead to formation of the other products identified in the reaction mixture (Scheme 2). For example, Cl-Tyr gave rise to both diCl-Tyr and Cl-HPAA on reaction with HOCl. The formation of diCl-Tyr from Cl-Tyr is analogous to the formation of Ac-diCl-Tyr from Ac-Cl-Tyr and diCl-Tyr from Cl-Tyr in proteins, whereas the formation of Cl-HPAA from Cl-Tyr is analogous to that of HPAA from tyrosine. When purified HPAA was reacted with HOCl, both Cl-HPAA and diCl-HPAA were formed (Fig. 4c).

Collectively, these data indicate that both HPAA and Cl-Tyr can form Cl-HPAA on reaction with HOCl. By analogy, we propose that both diCl-Tyr and Cl-HPAA are potential precursors for the formation of diCl-HPAA. These results are in agreement with a recent report that not only 3-bromotyrosine (a mono-brominated tyrosine) but also 3,5-dibromotyrosine (a dibrominated tyrosine) are formed from reaction of tyrosine with brominating agents such as eosinophil peroxidase (59).

Studies on the reaction mechanisms of HOCl have been the research interests of chemists and biochemists for many years. Formation of an aldehyde from an \( \alpha \)-amino acid by HOCl was first reported at the beginning of the last century (60, 61). Recent reinvestigation of the issue showed that formation of a chloramine at the \( \alpha \)-amino group is the first reaction step (24). It has been proposed that subsequent decarboxylation (20) of the chloramine leads to a short lived imine that undergoes spontaneous conversion to the aldehyde (7). Recent EPR studies have, however, revealed that chloramine decomposition can also be accompanied by formation of N-centered free radicals (26, 29).

With regard to the mechanism of chlorination of the aromatic ring by HOCl, several intermediates including \( \text{Cl}_2 \), \( \text{ClO}^- \), and \( \text{Cl}^- \) have been postulated as the chlorinating species (62). Domigan et al. (47) investigated the chlorination mechanism of tyrosyl residues in peptides by HOCl at physiological pH and...
suggested that HOCl initially reacted with an amine group of the peptide to form a chloramine that subsequently underwent an intramolecular reaction with a nearby tyrosyl residue to convert it to Cl-Tyr, rather than giving direct ring chlorination. Our data on the formation of Cl-HPAA from HPAA indicate clearly that ring chlorination can occur in molecules that do not contain an amine group, implying an additional chlorination mechanism; this is in accord with previous observations (62). Hazen et al. (51) have reported that Cl-Tyr is only formed from reaction of HOCl with free tyrosine at acidic pH and proposed that chlorine gas (Cl₂) was the chlorinating species. Our data, however, show that Cl-Tyr is indeed formed from free tyrosine at neutral pHe but can react further with HOCl, suggesting that Cl₂ may not be the only chlorinating species present in the system. Thus the detection of free Cl-Tyr in biological samples does not necessarily imply its generation in an acidic environment as postulated by Hazen and co-workers (51).

We have shown here that in a pathological circumstance in which neutrophil accumulation takes place, the mouse peritoneal abscesses, both Cl-Tyr and di-Cl-Tyr are formed on proteins, with a preponderance of the former by ~6–10-fold. Levels of both chlorinated species are much lower in human cataractous lenses, and advanced atherosclerotic plaques, as well as control tissues. This can be ascribed to the lower levels of neutral pH in these conditions, resulting in lower levels of Cl-Tyr and hence diCl-Tyr levels below our detection limit.

The physiological relevance of Cl-Tyr and di-Cl-Tyr formed on proteins by the action of HOCl remains to be defined. Modification of tyrosine at the active site of a protein may lead to inactivation of the protein. Aldehydes such as HPAA formed from reaction of HOCl with tyrosine are not inert species. They may react with other cellular targets such as proteins, lipids, and DNA. For instance, HPAA has been shown to react covalently with lysine residues in proteins to form Schiff base adducts (46). The generation of aldehydes from oxidation of amino acids by HOCl may represent an important mechanism for damage of biological targets at the sites of inflammation.

The toxicity of HOCl generated by activated phagocytes has been implicated in the pathogenesis of diseases ranging from atherosclerosis to ischemia-reperfusion injury and cancer (32–36). Studies on oxidation of human LDL by HOCl have revealed that the protein, rather than the lipid part of LDL, is the major site for HOCl damage (11). Exposure of LDL to HOCl results in transformation of human LDL into a high uptake form, which leads to intracellular accumulation of cholesterol and cholesterol esters when added to macrophages in culture (10). Formation of free radical species during decomposition of chloramine may result in secondary radical flux and damage to cellular components (26, 29, 63). Thus EPR studies indicate that chloramine formation and nitrogen-centered radicals are key species in HOCl-induced protein fragmentation (29) and can lead to the initiation of (secondary) lipid oxidation and antioxidant consumption in HOCl-treated LDL particles (63). CI-Tyr has been proposed as a unique and stable end product of HOCl oxidation and used as a marker for assessing the role of HOCl under oxidative stress (27, 48–50). However, our data show that neither Cl-Tyr nor HPAA is the end product from reaction of HOCl with tyrosine. We have shown here that diCl-Tyr should be measured along with Cl-Tyr to determine the extent of HOCl damage to host proteins in bacterial killing and in other inflammatory conditions characterized by a preponderance of neutrophils. Likewise, we propose that CI-HPAA and diCl-HPAA in addition to HPAA should be measured to assess the degree of oxidative damage by HOCl to free tyrosine. This is particularly important not only because of the intermediary nature of HPAA in the reaction but also due to the fact that other reactive species generated during exposure to ionizing radiation or Fenton systems can also oxidize tyrosine to HPAA (64–66). The chlorinated species of HPAA are therefore more indicative of HOCl involvement. One also has to be aware that HPAA, CI-HPAA, and diCl-HPAA are generated from free tyrosine only, not from tyrosyl residues in peptides or proteins. However, they have the potential to be covalently linked to proteins via reactions such as Schiff base formation.

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REFERENCES
1. Jesaitis, A. J., and Dratz, E. A. (1992) The Molecular Basis of Oxidative Damage by Leukocytes, CRC Press, Inc., Boca Raton, FL.
2. Kettle, A. J., and Winterbourn, C. C. (1988) Biochem. Biophys. Acta 1052, 379–385.
3. Kettle, A. J., Gedye, C. A., and Winterbourn, C. C. (1993) Biochem. Pharmacol. 45, 2351–2362.
4. Hazen, S. L., d’Avignon, A., Anderson, M. A., Hsu, F. F., and Heinecke, J. W. (1998) J. Biol. Chem. 273, 4997–5005.
5. Lamport, M. B., and Weiss, S. J. (1983) Blood 62, 645–651.
6. Arnold, J., Hammack, B., Wagner, M., Mueller, S., Arnold, K., and Grimm, E. (1990) Biochem. Biophys. Acta 991, 99–107.
7. Hasell, L. J., and Stocker, R. (1990) Biochem. J. 260, 165–172.
8. Hasell, L. J., van den Berg, J. J. M., and Stocker, R. (1994) Biochem. J. 292, 297–304.
9. Winterbourn, C. C., van den Berg, J. J. M., Roitman, E., and Kyuppers, F. A. (1994) Arch. Biochem. Biophys. 296, 547–553.
10. Karr, A. C., van den Berg, J. J. M., and Winterbourn, C. C. (1996) Arch. Biochem. Biophys. 332, 63–69.
11. Prutz, W. A. (1996) J. Biochem. Biophys. Methods 32, 125–135.
12. Prutz, W. A. (1998) Arch. Biochem. Biophys. 349, 183–191.
13. Vissers, M. C., and Winterbourn, C. C. (1995) Biochem. J. 307, 57–62.
14. Prutz, W. A. (1996) Arch. Biochem. Biophys. 332, 110–129.
15. Kettle, A. J., Roitman, E., and Stocker, R. (1997) Biochem. Biophys. J. 327, 275–81.
16. Kozlowski, M., and Kozlowska, T. (1994) J. Biol. Chem. 269, 10404–10413.
17. Van Tamele, E. H., Haarstad, V. B., and Orvis, R. L. (1968) Tetrahedron 24, 2357–704.
18. Zgliczynski, J. M., Stelmaszynska, T., Domanski, J., and Ostrowski, W. (1971) Biochim. Biophys. Acta 235, 419–424.
19. Heinecke, J. W., Li, W., Dachke, H. L., and Goldstein, J. A. (1993) J. Biol. Chem. 268, 4069–4077.
20. Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1996) J. Biol. Chem. 271, 1981–1867.
21. Anderson, M. M., Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 424–432.
22. Pereira, E. W., Hoyano, Y., Summons, R. E., Bacon, V. A., and Duffield, A. M. (1973) Biochim. Biophys. Acta 313, 170–177.
23. Hawkins, C. L., and Davies, M. J. (1998) J. Chem. Soc. Perkin Trans. 2, 1937–1945.
24. Heinecke, J. W., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2075–2081.
25. Thomas, E. L. (1979) Infect. Immun. 23, 522–531.
26. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
27. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
28. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
29. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
30. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
31. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
32. Neunhoeffer, S., Shigematsu, S., Kozlowski, M., and Kozlowska, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915–7922.
Marcinkiewicz, J., and Barzek, N. (1996) Acta Biochim. Pol. 43, 661–672
49. Kettle, A. J. (1996) FEBS Lett. 379, 103–106
50. Hazen, S. L., Crowley, J. R., Mueller, D. M., and Heinecke, J. W. (1997) Free Radical Biol. & Med. 23, 909–916
51. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) J. Clin. Invest. 98, 1263–1269
52. Van Reyk, D. M., Brown, A. J., Jessup, W., and Dean, R. T. (1995) Free Radical Res. 23, 533–535
53. Chung, B. H., Segrest, J. P., Ray, M. J., Brunzell, J. D., Hokason, J. E., Krauss, R. M., Beaudrie, R., and Cone, J. T. (1986) Methods Enzymol. 128, 181–209
54. Fu, S., Davies, M., Stocker, R., and Dean, R. (1998) Biochem. J. 333, 519–525
55. Fu, S., Dean, R., Southan, M., and Truscott, R. (1998) J. Biol. Chem. 273, 28603–28609
56. Kocher, M., PA, K., Farram, E., Abdul Majid, K., Pinlay-Jones, J., and Gezy, C. (1996) Infect. Immun. 64, 1342–1350
57. Dean, R. T., Fu, S., Gieseg, S., and Armstrong, S. G. (1996) in Free Radicals: A Practical Approach (Punchard, N., and Kelly, F., eds) pp. 171–183, Oxford University Press, Oxford
58. Hensley, K., Maidt, M., Pye, Q., Stewart, C., Wack, M., Tabatabaie, T., and Floyd, R. (1997) Anal. Biochem. 251, 187–185
59. Wu, W., Chen, Y., d’Avignon, A., and Hazen, S. (1999) Biochemistry 38, 3538–3548
60. Dakin, H. (1917) Biochem. J. 11, 79–95
61. Wright, N. C. (1936) Biochem. J. 30, 1661–1667
62. Swain, C. G., and Crist, D. R. (1972) J. Am. Chem. Soc. 94, 3195–3200
63. Hazell, L., Davies, M. J., and Stocker, R. (1999) Biochem. J. 339, 489–495
64. Garrison, W. M. (1968) Curr. Top. Radiat. Res. 4, 43–94
65. Garrison, W. M. (1987) Chem. Rev. 87, 381–398
66. Stadtman, E. R. (1993) Annu. Rev. Biochem. 62, 797–821
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