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Hypochlorous Acid

(HOCl)

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Abbreviations:

ERK, extracellular signal-regulated kinase
ESMS, electrospray mass spectroscopy
GSH, glutathione
GSSG, glutathione disulfide
GC-MS, gas chromatography-mass spectrometry
HPLC, high performance liquid chromatography
HOCl, hypochlorous acid
LDL, low-density lipoproteins
MPO, myeloperoxidase
NADPH, nicotinamide adenine dinucleotide phosphate
NF-kB, nuclear factor kappa B
OCl^-, hypochlorite

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Abstract

Hypochlorous acid (HOCl) is a powerful oxidant generated by the enzyme myeloperoxidase (MPO) from hydrogen peroxide (H_2O_2) and chloride (Cl^-) during the respiratory burst in phagocytic white blood cells. It plays an important role in the immune host defense by killing foreign pathogens ingested by phagocytes. HOCl exerts its antimicrobial function by producing lethal oxidizing species to destroy critical bacterial components. HOCl reacts readily with a wide range of biomolecules, including amines, lipids, thiols and proteins. HOCl can also produce $HO^{\bullet}$. Excessive production of HOCl, however, leads to host tissue damage and has been associated with various inflammatory diseases, such as glomerulonephritis and atherosclerosis. This paper reviews the reactivity and properties of HOCl, as well as its mechanism of action and methods of detection.

Introduction

In 1935 Baldridge and Gerard [1] discovered the "respiratory burst" when they observed that phagocytes exposed to bacteria underwent a dramatic but transient increase in the uptake of oxygen. The purpose of this sharp increase in oxygen consumption *via* the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex was not realized until 1961 when Iyer and colleagues [2] showed that phagocytic cells produced H_2O_2 in the presence of foreign pathogens. Klebanoff *et al*. [3] completed the story in 1967 with the finding that a heme enzyme myeloperoxide (MPO), which is stored in primary azurophilic granules of neutrophils, monocytes and certain types of macrophages [4], used H_2O_2 and Cl^- to manufacture a powerful microbicial reagent, HOCl. After ingesting bacteria, phagocytes secrete MPO into phagolysosomal compartment so that MPO can catalyze the formation of HOCl from H_2O_2 and Cl^- (**Figure 1**) [4]. As a potent chlorinating and oxidizing agent, HOCl is considered a critical line of defense against invading pathogens [1]. Indeed, HOCl is capable of killing bacteria within milliseconds (< 100 ms) [5] by targeting bacterial membrane transport systems [5], electron transport chains, and the adenine nucleotides (AMP, ADP, ATP) for oxidative degradation [6,7].

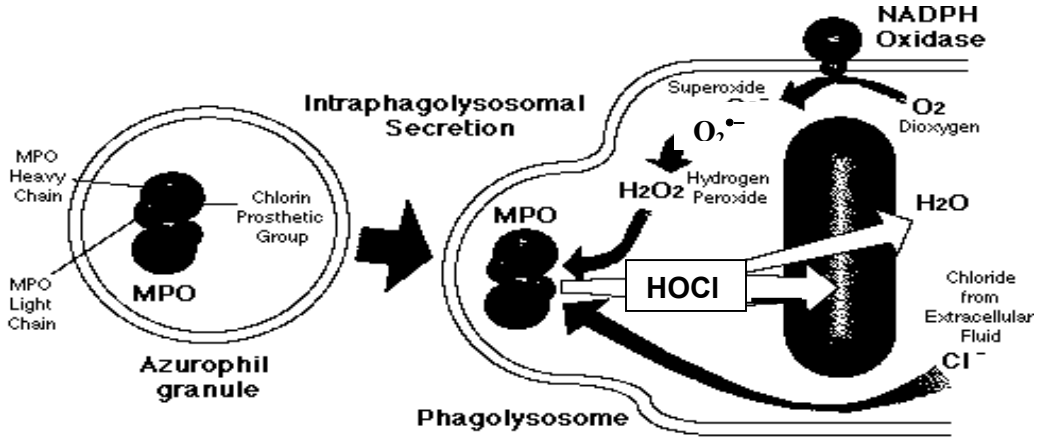

Figure 1. MPO is secreted into the phagolysosome where it produces HOCl to kill bacteria [4].

Properties of HOCl

HOCl is a powerful two-electron oxidizing agent with a molecular weight of 52.46 [1]. HOCl is generated by the MPO compound I (MPO-I)-H_2O_2-Cl^- system in activated phagocytes based on the following reactions [8,9,10]:

$$\text{MPO} + \text{H}_2\text{O}_2 \underset{k_{-1} = 58\ \text{s}^{-1}}{\overset{k_1 = 3.3 \times 10^7\ \text{M}^{-1}\ \text{s}^{-1}}{\leftrightarrows}} \text{MPO-I} + \text{H}_2\text{O} \qquad \text{(Reaction 1)}$$

$$\text{MPO-I} + \text{Cl}^- \underset{k_{-2} = 1.9 \times 10^5\ \text{s}^{-1}}{\overset{k_2 = 2.2 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}}{\leftrightarrows}} \text{MPO-I-Cl}^- \qquad \text{(Reaction 2)}$$

$$\text{MPO-I-Cl}^- \overset{k_3 = 5.2 \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}}{\rightarrow} \text{MPO} + \text{HOCl} \qquad \text{(Reaction 3)}$$

In this overall reaction, MPO-I-Cl plays the role of a chlorinating intermediate [8]. HOCl has a p*K*a of 7.6 so it is considered a weak acid. As such, HOCl undergoes partial dissociation to generate a hydrogen ion (H^+) and a hypochlorite ion (OCl^-) as shown in Reaction 4 [11]. HOCl is more reactive than its conjugate base, OCl^-, because of its ability to penetrate cell walls [12].

$$\text{HOCl} \leftrightarrows \text{H}^+ + \text{OCl}^- \quad \text{p}K\text{a} = 7.6 \qquad \text{(Reaction 4)}$$

The decomposition of HOCl generates chlorine gas (Cl_2) as shown in the following reaction [13]:

$$\text{HOCl} + \text{H}^+ + \text{Cl}^- \rightarrow \text{Cl}_2 + \text{H}_2\text{O} \qquad \text{(Reaction 5)}$$

Chlorine gas, like HOCl, is toxic to biological molecules. Indeed, Cl_2 is used by neutrophils as the chlorinating intermediate in the oxidation of lipids, such as cholesterol and low-density lipoproteins (LDL) [13].

Reactivity of HOCl

A. Thiol Compounds

As a powerful oxidizing agent, HOCl can react with many oxidizable functional groups but it most readily reacts with thiol compounds, such as glutathione (GSH) and cysteine [14]. Until recently, it was suspected that HOCl exerted its oxidizing function predominantly at the cell membrane but recent studies suggested that HOCl can penetrate the cell to react with intracellular species like GSH [15]. The rate constant for the reaction between HOCl and GSH is $10^7\ M^{-1}s^{-1}$ [14]. Because GSH is a major cellular antioxidant and a prime target of HOCl, it was important to characterize the products of the reaction between HOCl and GSH. Brennan and Winterbourn (16) used a combination of high performance liquid chromatography (HPLC) and electrospray mass spectrometry (ESMS) to study the products of GSH oxidation by HOCl. In addition to glutathione disulfide (GSSG), the reaction between GSH and HOCl generated two more major products with molecular masses 337 Da and 644 Da. Using HPLC, these two novel products were first identified as Peak A (sulfonamide; GSO_2NH) and Peak B (thiolsulfonate; GSO_2SG) and then their masses were determined by ESMS (**Figure 1**). The 337 Da product lacked free thiol and amine groups and was characterized as internal sulfonamide. The 644 Da product lacked free thiol groups but had amine groups and was identified as GSH thiolsulfonate

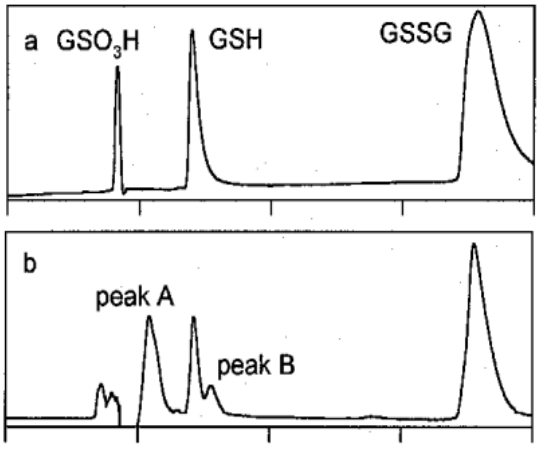

Figure 1. Product characterization of the reaction between GSH and HOCl. A) GSH, GSSG and GSO_3H Standards; B) reaction between 6 mM HOCl and 10 mM GSH at pH 7.4 [16].

B. Chloramines

Reaction of HOCl with amines ($k \approx 10^5\ M^{-1}\ s^{-1}$) results in the production of chloramines [17]:

$RNH_2 + HOCl \rightarrow RNHCl + H_2O$ (Reaction 6)

Chloramines, like HOCl, are also oxidants as evidenced by their ability to oxidize biomolecules like thioethers and heme proteins [17,18]. Chloramines also exhibit high reactivity toward thiols and methionine but they are several orders of magnitude less reactive than HOCl. The cytotoxicity of chloramines varies based on their structure and their ability to traverse membrane channels and gain access inside of cells [19,20]. Charged species, like taurine chloramine (Tau-Cl; $^{-}SO_3CH_2CH_2NHCl$) are cell impermeable while others, like glycine chloramine (Gly-Cl; **Figure 2**), readily penetrate the cell to interact with intracellular constituents [21].

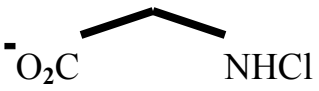

Figure 2. Structure of glycine chloramine (Gly-Cl).

Thus, depending on their cell permeability, chloramines are able to oxidize a wide range of intracellular or extracellular constituents. Tau-Cl, for example, was shown to oxidatively activate extracellular signal-regulated kinase (ERK) and inhibit p53 activation and nuclear factor kappa B (NF-kB) in various cell types [21]. However, the exact mechanism by which Tau-Cl regulates these cell signaling pathways is not yet determined [21]. Chloramines can induce cell cycle arrest and apoptosis or suppress proliferation in different cell models [22,23,24] so, in addition to their role as oxidants, chloramines are also considered important regulators of cell metabolism.

C. Chlorohydrins

HOCl also forms chlorohydrins ($k = 3.3 \times 10^5$ M^{-1} s^{-1}) by attacking double bonds (C=C) of unsaturated lipids, such as cholesterol. Cholesterol chlorohydrin formation in the presence of

HOCl has been demonstrated in different cells, including erythrocytes and mammary carcinoma cells [25]. The addition of HOCl to C=C double bond in cholesterol yields three different positional and steric isomers of chlorohydrins: α-chlorohydrin, β-chlorohydrin, and chlorohydrin-3 [26]. The formation of chlorohydrins is a two-step process (**Figure 3**). In the first step, one HOCl molecule is added to the C=C double bond with Cl^+ ion acting as the electrophilic molecule. In the second step, the remaining OH^- species is added to form the chlorohydrin. Because they are more polar than the parent lipids, chlorohydrins have the potential to damage cell membranes and induce lysis, which could be critical in phagocyte-induced toxicity [26].

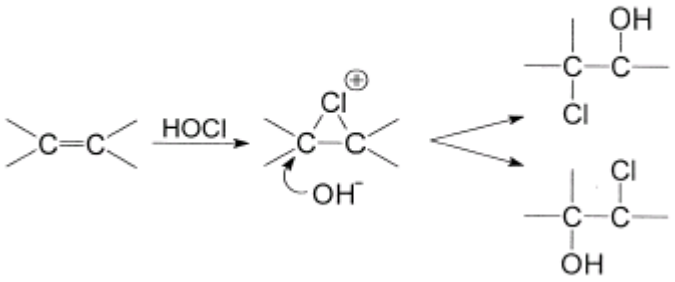

Figure 3. Chlorohydrin formation by HOCl [26].

D. Hydroxyl Radical

When HOCl reacts with superoxide radical ($O_2^{\bullet -}$) and iron (Fe^{2+}) ions, it produces hydroxyl radical ($HO^{\bullet}$) according to the following reactions:

$HOCl + O_2^{\bullet -} \rightarrow O_2 + Cl^- + HO^{\bullet}$ $k = 7.5 \times 10^6\ M^{-1}\ s^{-1}$, pH 5.0 [15] (Reaction 7)

$HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + HO^{\bullet}$ $k = 1.7 \times 10^4\ M^{-1}\ s^{-1}$, pH 4.0 [14] (Reaction 8)

The ability of $O_2^{\bullet -}$ and ferrous ions to convert HOCl into a more reactive species, $HO^{\bullet}$, demonstrates that $HO^{\bullet}$ is a major source of free radicals in a biological system [15].

Detection of HOCl

Because HOCl is a potent oxidant, the use of oxidation products as biomarkers of HOCl would be optimal. Unfortunately, oxidative reactions involving HOCl are relatively fast and nonspecific so oxidized products should not be used as target biomarkers [9,27]. In addition to being a strong oxidant HOCl is also an effective chlorinating species. Even though chlorinated products are produced at a comparably slower rate than oxidizing products, they are considered better biomarkers. Chlorinated products are also more specific biomarkers since chlorine is incorporated into the target molecule to more accurately detect HOCl. Some examples of chlorinated products that are used in detection of HOCl include chlorinated tyrosines, such as 3-chlorotyrosine and 3,5-dichlorotyrosine, formed when proteins react with HOCl [**Figure 4**; 11].

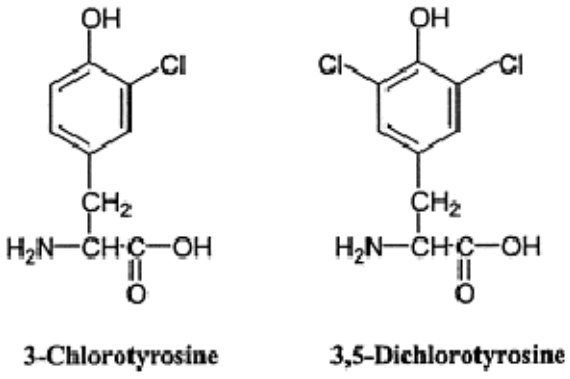

Figure 4. HOCl reacts with tyrosine residues to generate chlorinated tyrosines [11].

B. Gas Chromatography-Mass Spectrophotometry (GC-MS)

This is a method used to quantify tissue levels of 3-chlorotyrosine, which is a unique product formed during oxidation of peptides by HOCl. In this assay, the GC component separates the

sample mixture into pulses representing HOCl-derivatized chemical, 3-chlorotyrosine. The MS component then identifies and quantifies the levels of 3-chlorotyrosine. This method is highly sensitive as it detects between 100-300 attomoles in injected sample (**Figure 5**). It is also highly reproducible as evidenced by the inter- and intra-sample coefficients of variance being <3% (11).

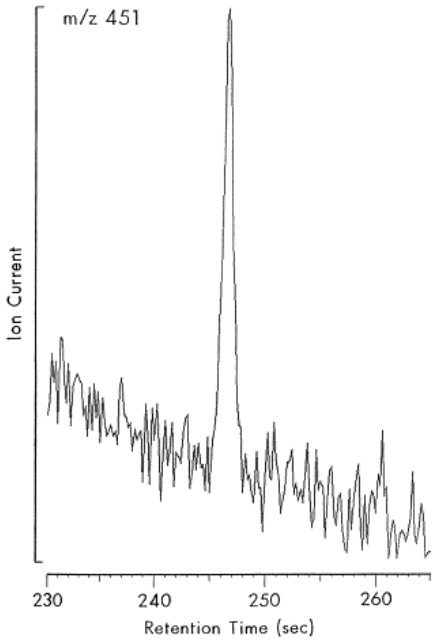

Figure 5. Detection of 3-chlorotyrosine (310 attomoles) using negative ion chemical-ionization GC-MS. The n-propyl per-HFB derivative of 3-chlorotyrosine (310 attomoles) in a tissue sample was examined at m/z 451 in the selected ion-monitoring mode [11].

A. High Performance Liquid Chromatography (HPLC)

This method, in combination with electrospray mass spectrophotometry (ESMS), has been used to characterize the products formed from the reaction between HOCl and GSH, including GSSG, sulfonamide and thiolsulfonate. In this method, the products are separated on a column and their mobilities compared to mobilities of standards (see **Figure 1)** [16]. HPLC fractions are then collected and analyzed further using electrospray-MS in both positive- and negative-ion modes. Unlike most other oxidized products, sulfonamide is specific to HOCl-mediated oxidization of

GSH and thus could be considered a useful biomarker of HOCl [16]. The potential for sulfonamide as the HOCl biomarker needs to be further explored however.

C. Immunocytochemistry

Antibodies against HOCl-oxidized proteins and HOCl-oxidized LDL have been generated. Malle *et al*. (4) generated polyclonal and monoclonal anti-human antibodies against HOCl-oxidized LDL because HOCl was shown to oxidize LDL and oxidized LDL is now known to contribute to atherogenesis. The polyclonal antibodies, but not monoclonal antibodies, were found to cross-react with 4-hydroxynonenal-, malodialdehyde- and Cu^{2+}-oxidized LDL. Monoclonal antibodies specifically bound to HOCl-oxidized LDL, thus confirming HOCl involvement in atherosclerotic plaque formation [4]. Antibodies against HOCl-oxidized proteins work based on the same principle except these antibodies are used to identify peptide injury induced by HOCl. In fact, antibodies against HOCl-oxidized proteins have already been used in study of HOCl-modified proteins in human kidney disease (glomerulonephritis) [28]. These studies identified interstitial and atrophic epithelial MPO-containing cells of diseased kidneys, therefore confirming the participation of MPO-H_2O_2-Cl^- system in kidney disease [28].

Summary

HOCl is a potent oxidant found in phagocytic cells, such as neutrophils and monocytes. It is generated by MPO during the oxidative burst of phagocytes. On one hand, HOCl plays an important role as the primary microbicidal reagent after phagocytosis. On the other hand, excessive production of HOCl has been implicated in numerous diseases, especially atherosclerosis, in which HOCl oxidizes LDL and converts it into high-uptake atherogenic form.

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