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Review

Measuring chlorine bleach in biology and medicine ☆

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ABSTRACT

Background: Chlorine bleach, or hypochlorous acid, is the most reactive two-electron oxidant produced in appreciable amounts in our bodies. Neutrophils are the main source of hypochlorous acid. These champions of the innate immune system use it to fight infection but also direct it against host tissue in inflammatory diseases. Neutrophils contain a rich supply of the enzyme myeloperoxidase. It uses hydrogen peroxide to convert chloride to hypochlorous acid.

Scope of review: We give a critical appraisal of the best methods to measure production of hypochlorous acid by purified peroxidases and isolated neutrophils. Robust ways of detecting it inside neutrophil phagosomes where bacteria are killed are also discussed. Special attention is focused on reaction-based fluorescent probes but their visual charm is tempered by stressing their current limitations. Finally, the strengths and weaknesses of biomarker assays that capture the footprints of chlorine in various pathologies are evaluated.

Major conclusions: Detection of hypochlorous acid by purified peroxidases and isolated neutrophils is best achieved by measuring accumulation of taurine chloramine. Formation of hypochlorous acid inside neutrophil phagosomes can be tracked using mass spectrometric analysis of 3-chlorotyrosine and methionine sulfoxide in bacterial proteins, or detection of chlorinated fluorescein on ingestible particles. Reaction-based fluorescent probes can also be used to monitor hypochlorous acid during phagocytosis. Specific biomarkers of its formation during inflammation include 3-chlorotyrosine, chlorinated products of plasmalogens, and glutathione sulfonamide.

General significance: These methods should bring new insights into how chlorine bleach is produced by peroxidases, reacts within phagosomes to kill bacteria, and contributes to inflammation. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

Chlorine bleach or hypochlorous acid is exceptionally toxic. It is produced by white blood cells and kills all types of bacteria. At the right dose, it is also lethal to our own cells. Hypochlorous acid is toxic because it oxidizes many biological molecules. Its tendency to bleach chemicals was first noticed by the great Swedish chemist Carl Wilhelm Scheele who discovered chlorine in 1774. He was also aware of its toxicity but failed to appreciate the dangers of chlorine gas, dying young after succumbing to its vapours [1]. Even though the antiseptic properties of chlorine were realised early, Semmelweis had to plead with surgeons to sanitize their operating theatres with bleach [2]. No one needed to be convinced of its toxicity during World War 1. Ironically, while infected wounds were cleansed with Dakin's dilute solution of hypochlorite, the toxic fumes of chlorine gas were blown across the battlefields of Europe to asphyxiate soldiers [3,4]. At this time, chlorine was beginning to be used in municipal water supplies to eradicate water borne diseases from urban populations. In 1967 Klebanoff discovered that the enzyme myeloperoxidase uses hydrogen peroxide to oxidize chloride to a potent bactericidal agent [5]. It was then obvious that chlorine bleach must be a leading participant in our battle against infections and the sequelae of inflammation. From that time, methods with ever increasing sophistication have been developed to explore the biology and pathology of chlorine bleach. In this review we will outline the basic chemistry and biochemistry of hypochlorous acid, and describe the most robust methods for measuring its production by peroxidases *in vitro*, within cells of the innate immune system, and at sites of inflammation.

Neutrophils, the most abundant white blood cells in the circulation of humans, are the major source of hypochlorous acid in our bodies [6,7]. When stimulated, they undergo a burst of respiration in which oxygen is reduced to superoxide by the NADPH oxidase that is assembled on their plasma membrane. Neutrophils contain rich stores of the haem enzyme myeloperoxidase. It uses superoxide and hydrogen peroxide to convert chloride to hypochlorous acid [8]. When a neutrophil ingests and kills a bacterium, hypochlorous acid is produced within the phagocytic vacuole that encases the bacterium (Fig. 1A) [6,7,9]. In diseases

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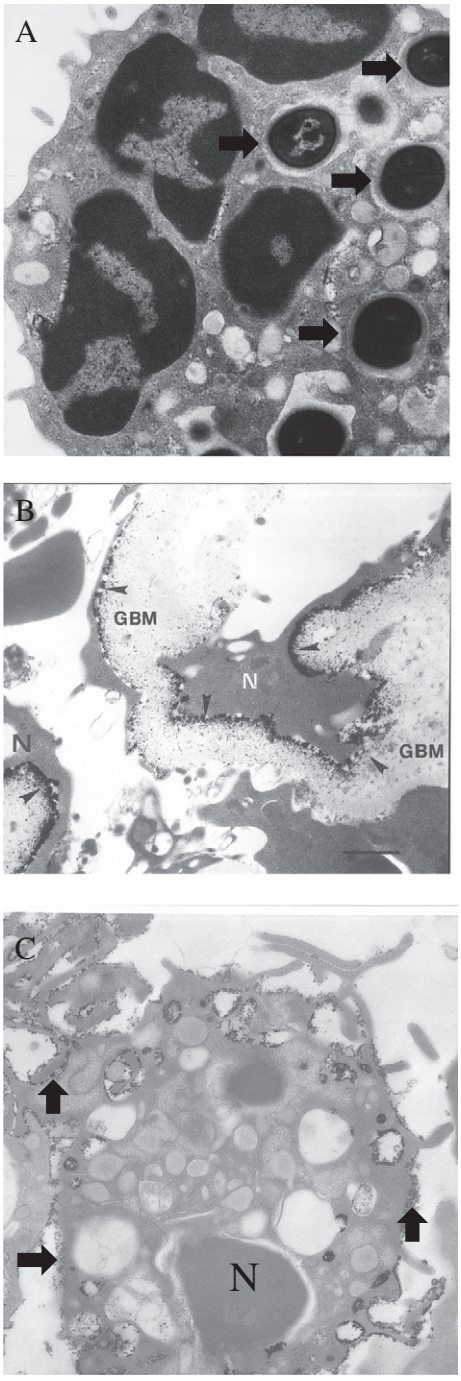

Fig. 1. Activation of neutrophils. (A) Neutrophils phagocytosing bacteria as indicated by arrows. "This research was originally published in *Blood*. Hampton MB, Kettle AJ and Winterbourn CC, Inside the neutrophil phagosome. *Blood*. 1998;92:3007–3017. © the American Society of Hematology." [173] (B) Neutrophils (N) producing hydrogen peroxide when adherent to basement membrane (GBM) and when (C) stimulated by phorbol myristate acetate. Hydrogen peroxide was visualized by the electron-dense deposits of cerium (see arrows) [10].

such as rheumatoid arthritis, neutrophils are activated by immune complexes on host tissue where they undergo frustrated phagocytosis and direct hypochlorous acid against the tissue [10] (Fig. 1B). Soluble stimuli cause the release of myeloperoxidase and hydrogen peroxide to the extracellular environment where hypochlorous acid will react with a variety of biological nucleophiles (Fig. 1C) [11]. Monocytes and macrophages also contain myeloperoxidase but in much lower amounts than neutrophils.

Hypochlorous acid should be viewed as a doubled-edged sword in biology. Its extreme toxicity toward virtually all bacteria is a boon for host defence but its facile reactivity with myriad biological molecules makes it a dangerous oxidant during inflammation. We are also learning that hypochlorous acid may play another vital role in biology by tying strands of collagen IV together in an indispensable structural cross-linking reaction [12]. This reaction is catalysed by peroxidasin 1, also known as vascular peroxidase 1 [13]. The methods outlined in this chapter will be valuable in elucidating this newly discovered use of hypochlorous acid and how peroxidasin 1 contributes to normal physiology and exacerbates inflammation [14,15].

2. The chemistry of chlorine bleach

Chlorine bleach is an ephemeral species that often leads to a non-specific footprint when it reacts with biomolecules. Consequently, detecting this fleeting oxidant in biological systems requires an appreciation of its potential chemistry. When the native or ferric form of myeloperoxidase reacts with hydrogen peroxide it forms the redox intermediate Compound I [16], which has a two electron reduction potential of 1.16 V [17]. Thus, Compound I is more likely to oxidize chloride to hypochlorous acid ($E^{0\prime}$HOCl/Cl^- 1.28 V) than molecular chlorine (Cl_2/Cl^- 1.39 V) [18]. However, the odour of chlorine gas wafting from solutions of myeloperoxidase, chloride and hydrogen peroxide [19], and its identification by mass spectrometry [20], demonstrates that under physiological conditions hypochlorous acid is in equilibrium with molecular chlorine. Dichlorine monoxide (Cl_2O) is another species of chlorine that is relevant to the understanding of the biological chemistry of hypochlorous acid. These reactive chlorine species form an overall equilibrium with hypochlorite ($^-$OCl), which is balanced by pH and the concentration of chloride (reactions 1, 2 & 3).

$$Cl_2 + H_2O <------> HOCl + Cl^- + H^+ \quad (1)$$

$$HOCl <------> {}^-OCl + H^+ \quad (2)$$

$$2HOCl <------> Cl_2O + H_2O \quad (3)$$

The equilibrium constant for reaction 1 (1.3×10^{-3} M^2) [21] and the pKa of reaction 2 (7.44 at 37 °C) [22] determine that hypochlorous acid and hypochlorite are the major species present under physiological conditions (Fig. 2). At pH 7.4 the concentrations of hypochlorous acid and hypochlorite are approximately equal while molecular chlorine

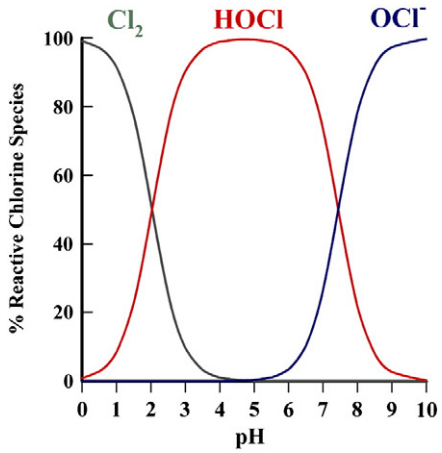

Fig. 2. The pH profile for reactive chlorine species. The relative concentrations of molecular chlorine (Cl_2; green), hypochlorous acid (HOCl; red) and hypochorite ($^-$OCl; blue) were calculated at 140 mM chloride using $K = 1.3 \times 10^{-3}$ M^2 for reaction 1 and a pKa of 7.44 for reaction 2.

accounts for less than 0.0002% of the reactive chlorine species. Even at pH 6.5, which occurs during inflammation in the airways [23], molecular chlorine makes up only about 0.003% of reactive chlorine. It accounts for 100 fold less at pH 7.8 which exists in neutrophil phagosomes when bacteria are being ingested and killed [24].

Hypochlorous acid is kinetically the most reactive two-electron oxidant produced in appreciable amounts in our bodies. Its reactivity with most substrates exceeds that of hydrogen peroxide, hydroperoxides, and peroxynitrite by several orders of magnitude [25]. The rates constants for the reactions of hypochlorous acid, however, vary over many orders of magnitude [26–28]. Thus, hypochlorous acid should be considered as a highly reactive but selective oxidant. It reacts overwhelmingly with cysteine and methionine residues ($k \sim 10^8\ M^{-1}\ s^{-1}$) and to a much lesser extent with lysine, tryptophan, terminal amines, and histidine ($k \sim 10^4$–$10^5\ M^{-1}\ s^{-1}$) [28,29]. Reaction with amine groups produces chloramines. These are weaker but more selective oxidants than hypochlorous acid [30,31]. When formed on protein residues they breakdown to form carbonyls as well as ammonia chloramines (NH_2Cl and $NHCl_2$) [32]. Amino acid chloramines have minimal antimicrobial activity whereas NH_2Cl and $NHCl_2$ have similar bactericidal potency to hypochlorous acid [32]. Alternative products of myeloperoxidase include hypobromous acid and hypothiocyanite, which are formed from the oxidation of bromide and thiocyanate, respectively [6,7]. For most amino acid residues hypochlorous acid reacts 30–100 fold slower than does hypobromous acid [33]. The exceptions are methionine and cysteine residues which react 10-fold faster with hypochlorous acid. Hypothiocyanite reacts rapidly with cysteine residues only [34].

The absolute rate constant for reaction of hypochlorous acid with tyrosine residues at pH 7.4 is only 40 $M^{-1}\ s^{-1}$, which indicates that direct chlorination under physiological conditions is unfavourable [28] (see Section 7). Although molecular chlorine is a minor form of reactive chlorine, it is a considerably stronger electrophile than hypochlorous acid [35,36]. Molecular chlorine should react at diffusion controlled rates with most biological nucleophiles. Hypochlorite is much less reactive than hypochlorous acid so that its reactions in biological systems can essentially be ignored [26].

For a particular reaction of chlorine bleach, the chlorine species that dominates depends on their relative rate constants and respective concentrations. For example, chlorination of tyrosine involves reaction with the phenolate form of this amino acid. The rate constant for hypochlorous acid with a phenolate is $2 \times 10^4\ M^{-1}\ s^{-1}$ whereas that for molecular chlorine will be at least $10^9\ M^{-1}\ s^{-1}$ [26]. Thus at pH 7.4, where the concentration of hypochlorous acid is 2.5×10^5 times that of molecular chlorine, both species would be expected to contribute to chlorination. However, with the sulfur containing amino acids the rate constants for both chlorine species approach the diffusion limit [26]. Hence, hypochlorous acid will be the major oxidant of these amino acids. In the biological milieu the reactions of hypochlorous acid with thiols and thioethers should predominate, so hypochlorous acid will govern the chemistry of reactive chlorine species. Only after methionine and cysteine residues have been oxidized, might the reactions of molecular chlorine become relevant. This could apply in the confined space of a phagosome where the most susceptible amino acids would be rapidly consumed due to the enormous flux of hypochlorous acid. Thus, reactions of molecular chlorine could explain why tyrosine trapped within red blood cell ghosts was chlorinated during phagocytosis [20].

The rate of reaction 3 is so slow ($k = 0.12\ M^{-1}\ s^{-}$) that dichlorine monoxide will not influence the reactions of hypochlorous acid *in vivo* [37]. However, this may not be so when reactions of hypochlorous acid are studied *in vitro*, especially at high concentrations of hypochlorous acid or when reaction 3 is catalysed by acids [38]. This is because dichlorine monoxide is a powerful electrophile and its rates of reaction can exceed those of hypochlorous acid by seven orders of magnitude [35,39]. Consequently, even though its concentration will be miniscule in solutions of hypochlorous acid ($K_3 = 1.5 \times 10^{-2}\ M^{-1}$ at 37 °C [38]), it could be the major reactive species when the target molecule is a weak nucleophile. For example, the rate constants for reaction of hypochlorous acid with backbone amides, arginine, tyrosine, phosphoryl-serine, aliphatic double bonds, and DNA are so low that dichlorine monoxide could contribute to their oxidation [40,41].

3. The enzymology of myeloperoxidase

Before attempting to measure hypochlorous acid production by myeloperoxidase, it is necessary to appreciate the complexity of the enzyme's reaction mechanisms. This awareness will help in optimizing enzyme activity by avoiding inactivation, and in understanding how inhibitors block production of hypochlorous acid. Initially hydrogen peroxide reacts with the native or ferric myeloperoxidase to form compound I (Fig. 3) [6,7,11,42]. This redox intermediate then oxidizes chloride to hypochlorous acid. Compound I is capable of oxidizing myriad other substrates because of its high reduction potential and the relatively large entrance to its active site. Bromide and thiocyanate are rapidly oxidized to their hypohalous acids via the same pathway as chloride [43,44]. Compound I also oxidizes many organic substrates to free radical intermediates. In the process, myeloperoxidase is converted to Compound II [45]. This latter reaction has a major influence on production of hypochlorous acid because compound II does not oxidize chloride and its reduction back to the ferric enzyme is often much slower than its formation. For example, hydrogen peroxide can reduce Compound I to Compound II but reacts with Compound II slowly [46]. Hence at high concentrations of hydrogen peroxide, hypochlorous acid production slows over time as Compound II accumulates [47]. Therefore, it is best to avoid using concentrations of hydrogen peroxide in excess of about 50 μM. If higher amounts of hydrogen peroxide are required, then it is preferable to use multiple additions of a low concentration or use glucose oxidase to generate a flux of hydrogen peroxide. In kinetic experiments, concentrations of hydrogen peroxide lower than 50 μM should not be used because its reaction with the enzyme may become rate limiting.

Accumulation of Compound II is strongly influenced by pH and the concentration of chloride [47,48]. At a high pH of 7.8 and low concentrations of chloride, Compound I is readily reduced to Compound II. However, at more acidic pH and high concentrations of chloride Compound II does not accumulate because its turnover is enhanced. *In vivo*, myeloperoxidase is likely to operate between pH 6.5 at sites of inflammation and pH 7.8 inside neutrophil phagosomes [24]. The concentration of chloride is likely to be about 100–140 mM. Hence, in assays designed to

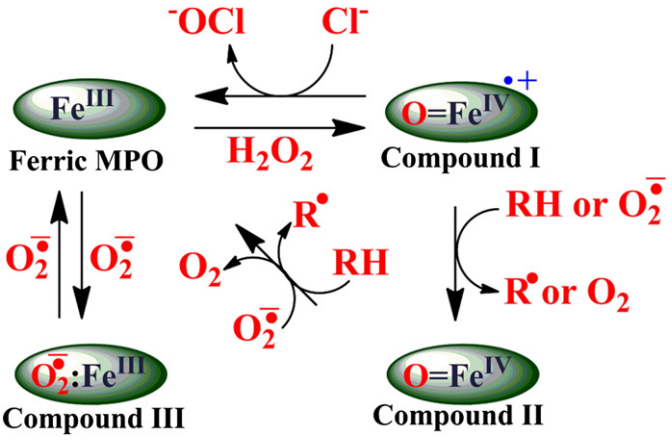

Fig. 3. The reactions of myeloperoxidase. Ferric MPO reacts with hydrogen peroxide to form Compound I. Compound I then oxidizes chloride, bromide, iodide or thiocyanate to their respective hypohalous acids in a two-electron reaction. Compound I is also reduced by one-electron donors, including hydrogen peroxide and organic substrate (RH) such as tyrosine, tryptophan, ascorbate, or serotonin to produce Compound II and a radical product. The classical peroxidation cycle is completed when substrates such as tyrosine, ascorbate and urate reduce Compound II back to the ferric enzyme. Superoxide reacts readily with ferric MPO, Compound I and Compound II.

measure hypochlorous acid production by purified myeloperoxidase, an initial choice of conditions is pH 7.4 and 140 mM sodium chloride.

Several other substrates promote accumulation of Compound II because they are good substrates for compound I but poor substrates for Compound II. These include tryptophan [49], melatonin [50], many non-steroidal anti-inflammatory drugs [51], and halogenated indoles [52]. Their presence in assays for hypochlorous acid production should be avoided or at least understood. Their effects can be attenuated by including a good substrate for Compound II in the reaction mixture at a concentration that minimizes its reaction with Compound I but allows it to readily reduce Compound II back to the active enzyme. Such substrates include tyrosine (20 μM) [51] and serotonin (5 μM) [50,53]. Any inhibitors of hypochlorous acid production that are less effective in the presence of tyrosine or serotonin are likely to act by promoting the accumulation of Compound II. This is by far the most common mechanism of inhibition of the enzyme.

Often myeloperoxidase is used in combination with xanthine oxidase. Xanthine oxidase with distilled acetaldehyde is employed as a source of hydrogen peroxide but it also generates superoxide [54]. Superoxide reacts with all the redox intermediates of myeloperoxidase [8] and in the presence of chloride converts the enzyme to Compound III [55]. Superoxide can either inhibit or enhance production of hypochlorous acid [55]. Thus, when using xanthine oxidase it is prudent to check how superoxide influences the activity of myeloperoxidase by measuring hypochlorous acid production in the presence and absence of superoxide dismutase.

4. Standardizing reagent hypochlorous ccid

The concentration of analytical grade hypochlorous acid is best determined by diluting it into 100 mM sodium hydroxide and then measuring the absorbance of hypochlorite at 292 nm (ε_{292} 350 M^{-1} cm^{-1}) [22]. The concentration of hypochlorous acid can be easily confirmed by adding it, with vigorous mixing on a vortex, to a solution of 5 mM taurine in phosphate buffer pH 7.4 to produce taurine chloramine. The chloramine is then reacted with 5-thio-2-nitrobenzoic acid (TNB) to bleach its yellow colour (ε_{412} 14,100 M^{-1} cm^{-1}) [56,57]. In this reaction one mole of taurine chloramine oxidizes two moles of TNB to give colourless 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). It is not advisable to react hypochlorous acid directly with TNB because it also oxidizes any DTNB that may be present in the original solution, giving an under-estimate of its true concentration. When using solutions of hypochlorous acid and chloramines it is imperative to mix them rapidly and effectively with solutions of target molecules. Otherwise, reactions will occur in localized areas giving stoichiometries and product distributions that are not representative of a homogenous solution.

5. Measuring hypochlorous acid production by myeloperoxidase

Hypochlorous acid cannot be monitored directly when it is produced by myeloperoxidase because it does not accumulate due to its rapid reactions with other constituents in solution including myeloperoxidase [58,59] and hydrogen peroxide [60]. Reaction with myeloperoxidase leads to enzyme inactivation [59]. Consequently, hypochlorous acid has to be scavenged by a reagent that gives a change in absorbance, fluorescence or chemiluminescence. Alternatively, it can be trapped with an amine such as taurine to give a stable chloramine. Whichever reagent is chosen to scavenge hypochlorous acid, it is imperative that the reaction is fast enough to prevent hypochlorous acid from reacting with other components of the system and that the reagent does not react with compound I to promote formation of compound II. Monochlorodimedon satisfies the first criterion as a detector [28] but promotes accumulation of compound II such that the chlorination activity is grossly under-estimated [45]. Detectors that show decreased production of hypochlorous acid when their concentration is increased should be avoided because they are likely to promote accumulation of compound II [28]. The following are the preferred assays for measuring hypochlorous acid production by purified myeloperoxidase. They are readily adapted to also measure production of hypobromous acid and hypoiodous acid by eosinophil peroxidase, lactoperoxidase, and peroxidasin 1.

5.1. The taurine chloramine assay

In this assay hypochlorous acid is scavenged by taurine (5 mM) to produce taurine chloramine, which retains the oxidizing capacity of hypochlorous acid but is considerably less reactive (Fig. 4, reaction 1) [56]. Reactions are best started by adding hydrogen peroxide (ε_{240} 43.6 M^{-1} cm^{-1}) [61] with mixing to the buffer containing taurine, myeloperoxidase and chloride. Taurine chloramine is stable at 37 °C for up to an hour. Solutions of taurine chloramine can also be placed on ice until measured where they are stable for hours. When other reagents are added to the buffer (e.g. potential inhibitors of myeloperoxidase), it should first be ascertained whether at the concentrations used they compete with taurine for reaction with hypochlorous acid. It should also be checked that they do not react with taurine chloramine. The main advantages of using taurine to trap hypochlorous acid is that it does not interfere with the activity of myeloperoxidase and the assay can be easily adapted to an ELISA plate format. The disadvantage of this assay is that hypochlorous acid production cannot be monitored continuously but rather the accumulation of taurine chloramine must be sampled at set intervals.

Taurine chloramine is easily assayed by measuring the extent to which it oxidizes 3,3′,5,5′-tetramethylbenzidine (TMB) in a reaction that is catalysed by iodide (Fig. 4, reactions 2&3) [62]. In this assay, taurine chloramine formed by myeloperoxidase is added to a 2 mM solution of TMB in the presence of iodide at pH 5.4. TMB is oxidized to a blue complex (ε_{650} ~ 30,000 $M^{-}1$ cm^{-1}) within five minutes [63]. Oxidation of TMB is much slower in the absence of iodide. Accurate determination of the concentration of taurine chloramine formed by myeloperoxidase is achieved by comparing results with a standard curve for reagent hypochlorous acid under the same conditions. The need for iodide as a catalyst gives this assay additional specificity for chloramines and distinguishes chloramines from bromamines [64]. The latter oxidize TMB independently of iodide. Additional sensitivity can be obtained by using dihydrorhodamine, which is oxidized by taurine chloramine in the presence of iodide to give highly fluorescent rhodamine [62]. The advantages of these assays are that a chromophore is produced rather than bleached, and that they are sensitive, specific and adaptable to a range of formats for measuring single or multiple samples.

Originally, taurine chloramine was measured by reacting it with 5-thio-2-nitrobenzoate (TNB) [57,65]. The advantages of using TNB are that it gives a sensitive and accurate readout of the concentration of taurine chloramine. Its chief disadvantage is that to obtain an accurate measurement, a prior knowledge of the taurine chloramine concentration is required so the initial concentration of TNB can be adjusted to give the largest possible loss in absorbance. This aspect complicates large scale analysis of hypochlorous acid production.

5.2. The NAD(P)H assay

Hypohalous acids react rapidly with NADH or NADPH to form halohydrins that absorb maximally at 274 nm (Fig. 5) [66]. NADH is a poor substrate for myeloperoxidase and does not interfere with enzyme activity. Hence NADH is an ideal detector for continuously monitoring the production of hypochlorous acid and hypobromous acid [67,68]. The absorbance of NADH (ε_{340} 6200 M^{-1} cm^{-1}) at 340 nm can also be monitored to determine the oxidation of NADH and thereby assess the stoichiometry of the reaction. The main disadvantage of this assay is that, depending on what other components are present, NADH may not always capture all of the hypochlorous acid that is generated. The

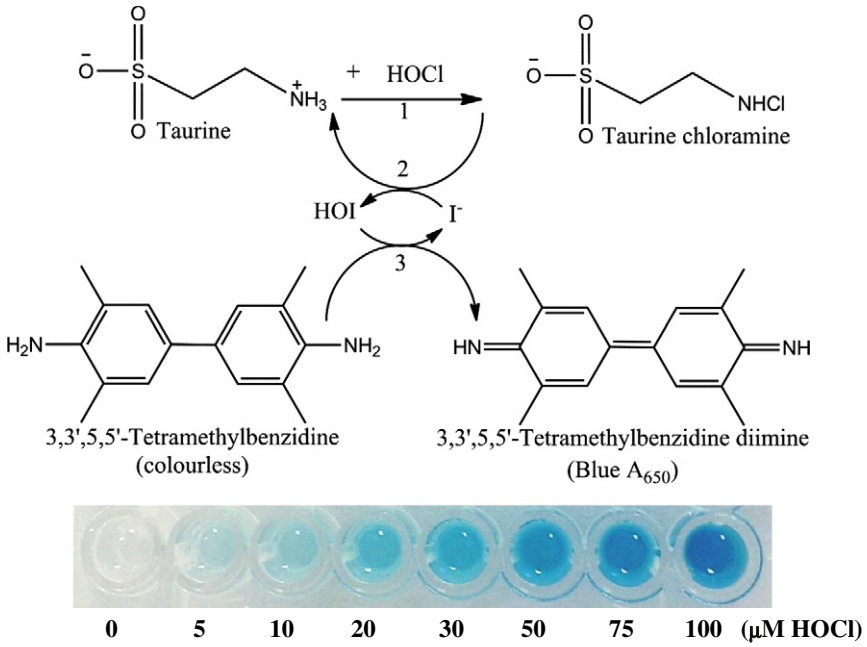

Fig. 4. The taurine chloramine assay. Hypochlorous acid is captured in reaction 1 where it forms taurine chloramine. It is subsequently assayed by adding it to iodide in the presence of 3,3′,5,5′-tetramethylbenzidine. The iodide is oxidized to hypoiodous acid (reaction 2), which in turn reacts with TMB to form the blue diimine (reaction 3). The ELISA wells show oxidation of TMB at increasing concentrations of hypochlorous acid (HOCl).

assay was recently used to demonstrated that ceruloplasmin is an uncompetitive inhibitor of myeloperoxidase with respect to its reaction with hydrogen peroxide [69].

5.3. The ascorbate assay

Ascorbate reacts rapidly with hypochlorous acid [41,70] and its consumption can be monitored at 266 nm (ε_{266} 15,000 M^{-1} cm^{-1}) [71]. Thus, the oxidation of ascorbate can be used to continuously monitor hypochlorous acid production by myeloperoxidase (Fig. 6) [71]. There are several important caveats that must be appreciated when using the ascorbate assay. Firstly, ascorbate is a substrate for myeloperoxidase, reacting readily with both Compound I and Compound II [72]. However, when used at 100 μM with 140 mM chloride, it competes poorly for oxidation by Compound I. Under these conditions essentially all the oxidation of ascorbate is via hypochlorous acid and little can be attributed to direct oxidation by myeloperoxidase [69]. In contrast, any Compound II that is formed in the ascorbate assay will be rapidly reduced back to the ferric enzyme. Hence, inhibitors that act by promoting the accumulation of Compound II will not be active in this assay. For example, at 420 nM, dapsone inhibits consumption of hydrogen peroxide by myeloperoxidase in the presence of chloride by 50% because it reduces Compound I to Compound II [51]. Yet in the ascorbate assay it failed to inhibit myeloperoxidase (Fig. 6). This facet of the ascorbate assay makes it ideal when trying to identify inhibitors that act in other ways besides promoting accumulation of Compound II. This is illustrated in Fig. 6 where the suicide substrate 2-thioxanthine [73] was a good inhibitor of myeloperoxidase in the presence of ascorbate. The assay was also recently employed to show that ceruloplasmin is a potent mixed type inhibitor of myeloperoxidase with respect to halides [69]. The maximum concentration of ascorbate that can be used in this assay is about 140 μM otherwise its absorbance exceeds the limits of

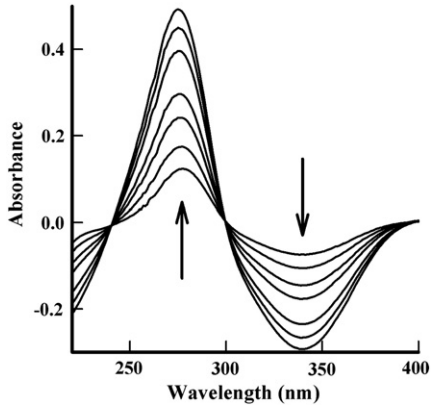

Fig. 5. Chlorination of NADH by myeloperoxidase. NADH (100 μM) was chlorinated by adding 50 μM hydrogen peroxide to 20 mM phosphate buffer pH 7.4 containing 140 mM sodium chloride and 20 nM myeloperoxidase. The difference spectra against NADH show the progress loss in absorbance at 340 nm due to oxidation of NADH and the increase in 275 nm due to formation of chlorohydrin. Arrows indicate direction of spectral changes over 5 min.

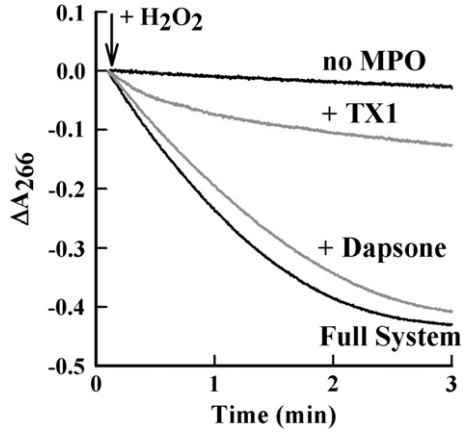

Fig. 6. Oxidation of ascorbate by myeloperoxidase. Ascorbate (100 μM) was oxidized by adding 25 μM hydrogen peroxide (arrow) to 10 mM phosphate buffer pH 7.4 containing 140 mM sodium chloride, 10 nM myeloperoxidase (MPO) and 100 μM diethyldiaminepenta acetic acid. The loss in absorbance at 266 nm was monitored with the full reaction system, in the absence of myeloperoxidase, in the presence of either 1 μM dapsone or 2 μM of the 2-thioxanthine TX1.

Beer's law. At this concentration it may not always out-compete other components in the system that react rapidly with hypochlorous acid.

5.4. Hydrogen peroxide consumption

The use of a hydrogen peroxide electrode to monitor hydrogen peroxide (ε_{240} 43.6 M^{-1} cm^{-1}) [61] consumption by myeloperoxidase has been described in detail previously [56]. In the presence of 140 mM chloride and methionine (1 mM to scavenge hypochlorous acid), it can be assumed to measure chlorination activity. Modern electrodes are easier to use than the original Clarke-type electrode and provide a sensitive measure of enzyme activity. They are useful for obtaining kinetic constants for the steady state production of hypochlorous acid and establishing how inhibitors affect enzyme activity [47,74]. However, components in the system can interfere with the electrode signal making it difficult to accurately record hydrogen peroxide consumption. Furthermore, the hydrogen peroxide electrode is useful for small scale experiments only.

Hydrogen peroxide consumption can also be measured with good sensitivity in a non-continuous assay using ferrous-iron oxidation of xylenol (FOX assay) [75]. This is a colorimetric assay where ferrous ammonium sulfate is oxidized by hydrogen peroxide leading to the formation of a blue-purple complex with xylene orange. The inclusion of sorbitol amplifies ferrous oxidation via formation of hydroperoxyl radicals, raising the sensitivity of the assay. When investigating reactions of myeloperoxidase in the presence of 140 mM chloride and 1 mM methionine, detection of hydrogen peroxide consumption by the FOX assay can be interpreted as a measure of hypochlorous acid production. In our laboratory we have found this to be a valuable assay that gives comparable inhibition data to that for other measures of the chlorination activity of MPO. This assay is amenable to testing a variety of reaction conditions and can be easily adapted to high throughput screening using a plate reader.

6. Measuring hypochlorous acid production by neutrophils

6.1. Detection of extracellular hypochlorous acid

In our experience the taurine chloramine assay is the method of choice for quantitative determination of extracellular production of hypochlorous acid by stimulated neutrophils [50,62,64,76,77]. Neutrophils are incubated in Hanks buffered saline solution containing 5 mM taurine at pH 7.4 and 37 °C and then stimulated with either phorbol myristate ester (PMA), formyl-Met-Leu-Phe or opsonized zymosan. After set time intervals, reactions are stopped by adding catalase (10 μg/ml) to remove residual hydrogen peroxide, cells pelleted by centrifugation, and the accumulated taurine chloramine detected using iodide-catalysed oxidation of TMB as described above. Normally, 2×10^6 neutrophils per ml will produce about 50 μM hypochlorous acid in 30 min when stimulated with PMA. The advantages of this assay are that it is simple, sensitive, and specific. The limitations are that it is not possible to continuously monitor the formation of hypochlorous acid and taurine chloramine may react with constituents in the reaction systems. This latter possibility should be assessed for each component in the system before commencing experiments with cells.

Oxidation of methionine to methionine sulfoxide can also be used to quantitatively determine hypochlorous acid production by neutrophils [78,79]. The rapid reaction of methionine with hypochlorous acid ensures that at sufficiently high concentrations of methionine all the hypochlorous acid can be trapped [28]. Also, methionine sulfoxide is stable and in the presence of excess methionine is relatively unreactive to further oxidation. The presence of contaminating methionine sulfoxide in purified methionine or artefactual oxidation of methionine can limit the sensitivity of this assay. A major drawback is that the detection of methionine sulfoxide requires a derivatisation procedure coupled with HPLC or direct analysis using liquid chromatography with mass spectrometry [78,79].

6.2. Detection of hypochlorous acid inside neutrophil phagosomes

It is necessary to measure hypochlorous acid inside neutrophil phagosomes to understand what factors are crucial for its efficient production, whether its production is compromised in certain immune defects, such as cystic fibrosis, and whether pathogenic bacteria may block its formation. It is not sufficient to just measure its extracellular production because conditions within phagosomes are vastly different to the surrounding milieu. Concentrations and fluxes of participants required to form and consume hypochlorous acid are markedly elevated in the tiny space available within the phagosomes [80]. For example, it has been estimated that the concentration of myeloperoxidase is relatively enormous at approx. 1 mM, the flux of superoxide incredibly fast at 2 mM/s, and the concentration of amino acid residues is about 1.5 M [80]. The concentration of chloride is likely to be similar inside and outside the phagosome but this has yet to be unequivocally established [81]. Kinetic modelling of oxidant production leads to predictions that under optimal conditions most of the superoxide generated by the NADPH oxidase inside phagosomes is converted to hypochlorous acid [80]. Furthermore, if it were to accumulate, the concentration of this oxidant should be in the hundreds of millimolar. Thus, it would be natural to expect that measurement of hypochlorous acid inside phagosomes should be a trivial task. Nothing could be further from the truth. This is because most of the hypochlorous acid is expected to react with neutrophil proteins inside the phagosome. Consequently its measurement is fraught with all the difficulties of detecting a transient oxidant that reacts by multiple routes and leaves the same chemical footprints as several other reactive oxygen species. The challenge of measuring hypochlorous acid inside neutrophil phagosomes requires a probe that captures enough oxidant to reflect its kinetics and extent of formation, and preferably retains chlorine as a molecular foot print.

Several approaches have been used to detect hypochlorous acid in phagosomes. In most studies bacteria ingested by neutrophils have been used as the probes for hypochlorous acid. Initially, it was shown that tyrosine residues in bacterial proteins are chlorinated to form the stable and specific product 3-chlorotyrosine that was detected using stable isotope dilution mass spectrometry [82,83]. Bacteria were grown with a heavy isotope of tyrosine to unequivocally demonstrate that the chlorinated product originated from the bacteria and not neutrophil protein [82]. This method has been used to show that there is a defect in phagosomal production of hypochlorous acid in neutrophils from patients with cystic fibrosis [84]. Its strengths are its high degree of sensitivity and specificity for reactive chlorine species. However, its weakness is that 3-chlorotyrosine is a minor product when hypochlorous acid reacts with proteins [85,86]. Therefore, detection of 3-chlorotyrosine will represent only a small percentage of the total hypochlorous acid produced. As a consequence it may not be a good barometer of subtle changes in oxidant generation.

To overcome the problem of a low yield biomarker, Rosen et al. used liquid chromatography with mass spectrometry to measure methionine sulfoxide in bacterial proteins after the bacteria had been ingested and killed by neutrophils [83]. Methionine sulfoxide was detected by an increase of 16 mass units in tryptic peptides of bacterial proteins that contained methionine. Approximately 50% of bacterial methionine residues were oxidized in a process that was reliant on the NADPH oxidase, myeloperoxidase and chloride. These results suggest that bacterial methionine sulfoxide is a good biomarker of hypochlorous acid production inside phagosomes. However, it should be remembered that methionine sulfoxide is not a specific for hypochlorous acid and other oxidants may contribute to the signal [87].

Expression of green fluorescent protein (GFP) in the cytosol of bacteria has been used to visualise hypochlorous acid production during killing of ingested micro-organisms [88,89]. GFP is highly sensitive to

hypochlorous acid but not to chloramines or other oxidants. The major conundrum with this approach is that bacterial viability was lost much more rapidly than the GFP was bleached. Chlorination of the fluorescent tyrosyl residue in GFP is expected to be slow, so it may not occur until the more reactive reductants inside the bacteria are consumed. Expression of GFP on the outside of bacteria may overcome this problem.

An alternative approach to the above methods is to couple a probe to a phagocytosable particle and subsequently measure its degree of oxidation or chlorination. In an example of this method fluorescein was conjugated to polyacrylamide microspheres via a reducible cystamine disulphide bond [90,91]. When phagocytosed it underwent almost complete conversion to chlorinated derivatives that were detected using liquid chromatography with mass spectrometry. Chlorination coincided with phagocytosis and the respiratory burst of the neutrophils. This method has the advantage of detecting a relatively abundant biomarker that is specific for hypochlorous acid. However, unlike the approaches described above using bacteria, it cannot be directly related to defects in killing of particular micro-organisms. A related method, in which dichlorodihydrofluorescein was conjugated to yeast, demonstrated the kinetics for hypochlorous acid production inside the phagosomes of a myeloid cell line [92]. Hypochlorous acid reacted with the probe to give a unique red-shifted fluorescence that was used to image oxidant production during phagocytosis.

6.3. Detection of hypochlorous acid using reaction-based fluorescent probes

In recent years numerous small molecule reaction-based indicators for hypochlorous acid have been ingeniously constructed to exploit the fluorescent properties of rhodamine, BODIPY, and fluorescein [93–103]. These probes have a reactive group that is oxidized by hypochlorous acid to unmask the fluorescent moiety (Fig. 7). They are stable to photo-bleaching and, given their high sensitivity, are ideal for monitoring hypochlorous acid production in biological systems. They are better probes than dichlorofluorescein and rhodamine because they have greater specificity and are less prone to artefactual oxidation [104]. The probes were used to visualize the production of hypochlorous acid within neutrophils and macrophages [97,99]. Co-localisation of fluorescence with the ingestible provides additional evidence that hypochlorous acid is produced inside phagosomes and therefore a component of the neutrophil's antimicrobial arsenal (Fig. 7).

Recently cadmium-selenide quantum dots were developed as a probe for hypochlorous acid to overcome some of these limitations because they exhibit superior photophysical properties [105]. The quantum dots had good sensitivity and specificity and were used to show production of hypochlorous acid in HL60 cells. However, their utility still needs to be examined in neutrophils.

The results to date with fluorescent probes demonstrate their exciting potential to monitor in real time the kinetics of hypochlorous acid production within neutrophil phagosomes and how this may be modulated. They also have potential to illustrate the temporal and spatial production of hypochlorous acid production by neutrophils and macrophages as they interact with immune complexes and host tissue during inflammatory pathologies. However, before being captivated by the visual splendour of these probes and their potential to reveal novel aspects of oxidative biology, their inherent shortcomings need to be understood. This will ensure that results obtained with them are interpreted with caution and new generations of probes are designed to overcome their current limitations. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in livings cells has been critically evaluated elsewhere in this series [104].

Although the probes have been reported to be specific for hypochlorous acid, the range of other oxidants tested against the probes has in general been limited to superoxide, hydrogen peroxide, hydroperoxides, singlet oxygen, hydroxyl radical, peroxyl radical, and peroxynitrite. Related halogen-derived oxidants such as hypobromous acid, chloramines, bromamines, and hypothiocyanite have not been assessed. Testing these species will be necessary to establish specificity because oxidants spawned from bromide are produced by both neutrophils and eosinophils [76,106]. Consequently, *in vivo* fluorescence could originate from either cell type. Also, chloramines retain some of the oxidizing potential of hypochlorous acid and slowly breakdown to release ammonia chloramine and ammonia dichloramine [32]. Therefore, the kinetics for the oxidation of the probes may be determined by the chemistry of the chloramines rather than the formation of hypochlorous acid. In

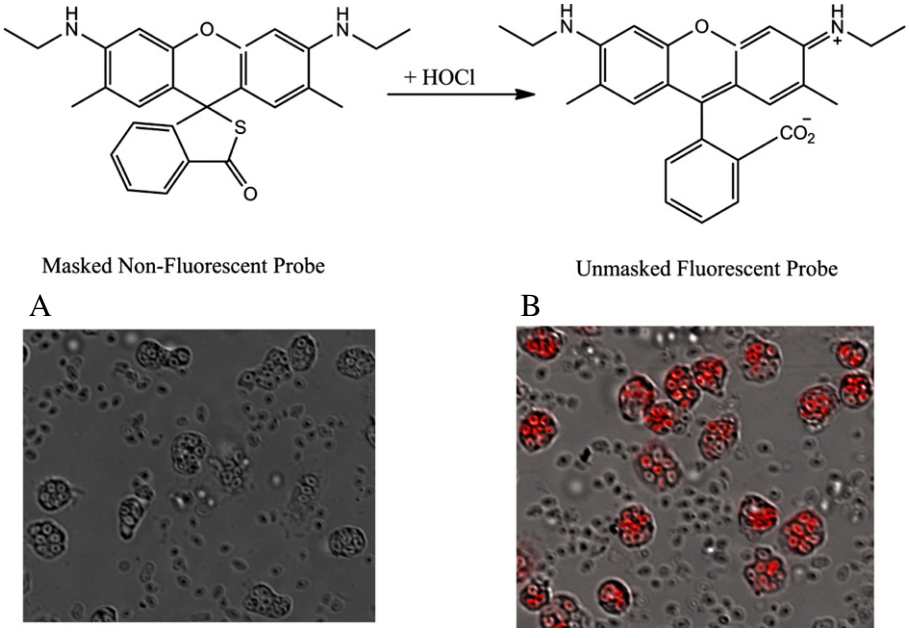

Fig. 7. Unmasking a reaction-based fluorescent probe for hypochlorous acid. Reaction of the probe R19-S with hypochlorous acid to produce its fluorescent product. Neutrophils (1×10^6/ml) were incubated in Hanks buffer pH 7.4 at 37 °C with 10 μM R19-S and stimulated with opsonized zymosan (2×10^7 particles/ml) in the (A) presence and (B) absence of the NADPH oxidase inhibitor 10 μM diphenyliodonium. Hypochlorous acid is visualized (B) by the red fluorescence associated with the phagocytozed zymonsan particles. The images were taken using an Olympus IX81 live cell imaging microscope with an XM10 camera (20 times objective) using CY3 and differential interference contrast.

contrast to the other oxidants, hypothiocyanite reacts selectively with thiols and seleno compounds but poorly with most other biological nucleophiles [107,108]. Hence, these probes, particularly the selenoether types [96], may be oxidized to a greater extent by hypothiocyanite than hypochlorous acid simply because the latter oxidant has multiple other targets.

Some alternative unmasked probes may also be prone to direct oxidation by myeloperoxidase without the need for hypochlorous acid production. The sulfonapthoaminophenyl fluorescein probe is a case in point [51]. This probe contains an aniline group that could be readily oxidized by myeloperoxidase. It also has the potential to be an inhibitor of hypochlorous acid production by promoting the accumulation of compound II. These aspects of reaction-based indicators should be thoroughly assessed before assuming myeloperoxidase oxidizes them efficiently via the production of hypochlorous acid.

Currently, there is limited information on the rates of reaction of the probes with hypochlorous acid. Rate constants for these reactions are needed to assess whether the probes will be major targets for hypochlorous acid within phagosomes or at sites of inflammation. Therefore, at this time they cannot be used to provide quantitative or even semi-quantitative data on the yields of hypochlorous acid produced by immune cells under various settings. Also, if the fluorescent yields are too low, they may not fully represent the kinetics of hypochlorous acid production. The probes with sulfur or selenoether [96] oxidisable moieties show promise as quantitative probes but those with alkene groups [109] are likely to be too unreactive to compete with biological targets.

Another issue with the use of the probes is that there is currently no way of determining the yield of the fluorescent species compared to the parent. Consequently, a high signal may result from either extensive oxidation of the probe or accumulation of large amounts of its oxidized form at a particular location in cells or tissue. The new generation of ratiometric fluorescent probes should assist in providing quantitative measurements via self-calibration of two emission bands [109,110]. When used critically, the current probes are useful for illustrating formation of hypochlorous acid inside phagosomes. Their future development will undoubtedly advance our understanding of how hypochlorous acid contributes to host defence and inflammation.

7. Finding evidence of hypochlorous acid production during inflammation

Neutrophils play a pivotal role in numerous pathologies. They combat infections, resolve inflammation by assisting in wound debridement [111], but exacerbate inflammation in diseases such as rheumatoid arthritis [112], cystic fibrosis [113], chronic obstructive pulmonary disease [114], gout [115], atherosclerosis [116], and sepsis [117]. There is also an increasing focus on how neutrophils are exploited by cancer cells to promote their own growth, invasion and metastasis [118]. Linking hypochlorous acid to these pathologies requires the use of specific and sensitive biomarkers that will unequivocally demonstrate its generation at sites of inflammation and that these molecular footprints are associated with disease severity. Their attenuation by drugs that target myeloperoxidase and alleviate disease will be the ultimate proof that hypochlorous acid has a causal role in a particular pathology.

An ideal biomarker for hypochlorous acid is a specific and stable product of its fast reaction with a biological molecule. This product should be amenable to sensitive and quantitative analysis. The most specific products are those that contain a chlorine atom. However, chlorination reactions of hypochlorous acid are much slower than its oxidation reactions [11]. Protein chloramines are likely to be abundant products but they react readily with reducing compounds, such as ascorbate, and thiols. They are also unstable and breakdown to generate protein carbonyls that are not specific to hypochlorous acid [86,119]. Chlorination of the vinyl ether moiety of plasmalogens produces a range of chlorinated metabolites [120,121]. Their chlorination is relatively favourable because the ether group alpha to the double bond facilitates the reaction compared to that for a simple double bond in lipids [122]. The latter also gives rise to chlorohydrins but via a slow reaction [123]. Direct chlorination of tyrosyl residues to form 3-chlorotyrosine is also slow [28]. In proteins, chlorination of tyrosyl residues most likely occurs via a juxtaposed chloramine [124,125]. The biological stability of 3-chlorotyrosine has been questioned because it can be metabolized to 4-hydroxyphenylacetic acid [126] or may be further chlorinated or nitrated [127,128]. As discussed above, NAD(P)H is readily chlorinated by hypochlorous acid to form chlorohydrins. However, given the relatively low concentration of NAD(P)H in cells, it is unlikely that they would be chlorinated at sites of inflammation. DNA bases are also chlorinated to give 8-chloroguanosine, 5-chlorocytosine, and 5-chlorouracil [129–134]. These reactions are relatively slow [135] and although 5-chlorouracil has been detected in inflammatory tissues [132,133], it was suggested that little hypochlorous acid can diffuse across the cytoplasm of cells and react with DNA to produce a useful biomarker [136]. However, chlorinated and brominated products of deoxyguanosine have been detected in urine of healthy individuals [137]. These levels were increased eight fold in diabetics. Thus, halogenated deoxyguanosines have potential as biomarkers of oxidative stress during inflammation.

Although oxidation reactions of hypochlorous acid are favourable, in general they do not furnish specific products [25]. Fortunately, there are some interesting exceptions. These involve the reactions of hypochlorous acid with thiols and methionine. In both cases the initial product is a sulfenyl chloride [138,139]. This normally hydrolyses to produce a sulfenic acid or sulfoxide, respectively. Sulfenic acids can react further to produce a disulfide [140]. However, when the sulfenyl chloride is in close proximity to a nucleophilic amine group, sulfenamides are also produced with good yields. These are further oxidized to sulfonamides. Although these products are not restricted to reactions of hypochlorous acid, few other biological oxidants produce them and, when measured in combination with myeloperoxidase, they provide a good degree of specificity. Hypochlorous acid was first shown to produce a sulfonamide when it reacts with glutathione [138]. Related sulfinamides were then found in the neutrophil protein calprotectin after it was oxidized by a modest dose of hypochlorous acid [141,142]. Oxidized calprotectin is present in sputum from asthmatics, which indicates this chemistry is relevant to neutrophilic inflammation [143]. Hypochlorous acid is likely to form these bonds within and between other proteins based on the susceptibility of cysteine containing peptides to form intra and intermolecular sulfenamides, sulfinamides and sulfonamides [144]. Methionine and peptides with N-terminal methionine residues afford the analogous compound dehydromethionine in good yields [145]. This is a cyclic product in which the sulfur atom of the methionine side chain becomes bonded to the N-terminal amine. Ubiquitin forms this product in high abundance as should other proteins with an N-terminal methionine [146]. Recently, it was found that upon reaction with hypochlorous acid the γ-glutamyl residues of glutathione disulfide are converted to 5-hydroxybutyrolactams [147]. These products were suggested to be specific and potential biomarkers for the activity of myeloperoxidase.

Given the various pros and cons of the potential biomarkers for hypochlorous acid, the following established methods are recommended for tracking its production *in vivo* and attempting to show its relevance to pathology.

7.1. Detection of 3-chlorotyrosine

Currently, measurement of 3-chlorotyrosine (Fig. 8) in proteins is the gold standard for detecting production of hypochlorous acid *in vivo*. Its strengths as a biomarker are that it is highly specific, there are very sensitive and robust methods using mass spectrometry for its detection [148,149], and its levels correlate strongly with the presence of myeloperoxidase. Normally proteins collected from sites of inflammation

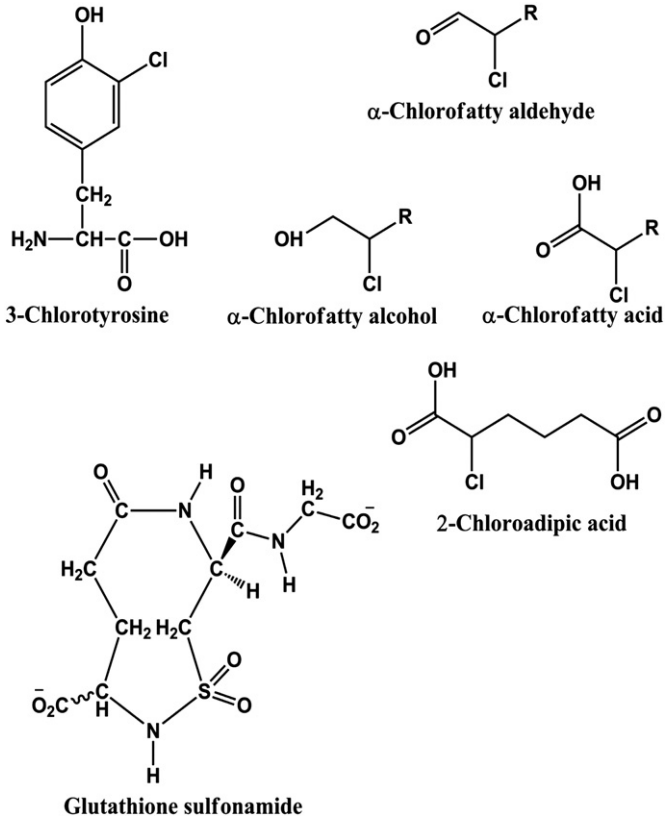

Fig. 8. Biomarkers for hypochlorous acid formed in its reactions with biomolecules. Chlorination of tyrosine residues produces 3-chlorotyrosine, while hypochlorous acid converts plasmalogens to α-chlorofatty aldehydes, alcohols, and acids, as well as 2-chloroadipic acid. Oxidation of glutathione produces glutathione sulfonamide as a minor but specific product.

are hydrolysed to liberate all the amino acids including 3-chlorotyrosine and tyrosine [148,149]. Methanesulfonic acid is the preferred acid for hydrolysis because artefactual halogenation of tyrosine is minimized compared to that observed with hydrochloric or hydrobromic acids [150]. Stable isotopes of 3-chlorotyrosine and tyrosine are included in the hydrolysis to check for artefactual chlorination of tyrosine. They also provide accurate quantitative analysis by accounting for loses during sample handling and diminution of signals due to ion suppression that may occur in the mass spectrometer. In the original method using gas chromatography, the amino acids were derivatized to make them volatile and then analysed based on their specific ion fragments plus the characteristic 3:1 ratio of isotopes for molecules containing a chlorine atom. The concentrations of 3-chlorotyrosine and tyrosine are calculated by comparing their signals to those for the known amounts of their stable isotopes that were added before hydrolysis. The level of 3-chlorotyrsine is then reported in μmoles per mol of tyrosine. In recent times liquid chromatography with mass spectrometry has superseded gas chromatography as the method of choice for 3-chlorotyrosine [151]. It has the advantage that it is unnecessary to derivatize the amino acids, which decreases the chance of chlorination during sample preparation. There are comparable levels of sensitivity between the two techniques. Other aspects of this stable isotope dilution assay are essentially the same as in the original method.

Detection of 3-chlorotyrosine was used to show that hypochlorous acid is produced in the plaques of individuals with atherosclerosis [149,152], joints of patients with rheumatoid arthritis [153], and in the airways preterm infants [154] and children with cystic fibrosis [155]. In each case the levels of 3-chlorotyrosine correlated with myeloperoxidase. Importantly, its levels were abrogated in myeloperoxidase-knockout animals when studying the role of the enzyme in mouse models of infection [156] and inflammation [157].

The major limitation with measuring 3-chlorotyrsoine/tyrosine in biological samples is that the signal is diluted out by all the tyrosine that is liberated from oxidized as well as unaffected proteins. To avoid this problem it is best to sample the physical site where myeloperoxidase is active and target proteins in these sites that are subjected to high doses of hypochlorous acid. Even greater sensitivity is achieved by analysing the tyrosine-containing peptides within these proteins that are most receptive to chlorination. This approach was used to show that high density lipoprotein (HDL) in atherosclerotic lesions contains high levels of 3-chlorotyrosine even when the signal in plasma was no higher than in healthy controls [158]. It was then demonstrated that apoliprotein A-1, the major protein in HDL, was selectively targeted for chlorination [151] and that specific tyrosine residues in the protein were the major sites for chlorination [159,160]. Subsequently, it was found the tryptic peptide LAEYAK from apo-A1 in HDL isolated from atherosclerotic lesions is highly chlorinated compared to plasma from the same patients [161]. This latter study highlighted the power of tandem mass spectrometry to detect 3-chlorotyrosine in selected proteins by monitoring specified tryptic peptides and their chlorinated forms. The degree of chlorination of these peptides can be used as a barometer to gauge the extent to which hypochlorous acid contributes to inflammatory tissue damage.

7.2. Detection of chlorinated products of plasmalogens

Plasmalogens are the major phospholipids of the plasma membranes of endothelial and vascular smooth muscle cells and cardiac myocytes. When hypochlorous acid reacts with their vinyl ether group it releases α-chlorofatty aldehydes (Fig. 8), such as 2-chlorohexadecanal [121]. These products are then oxidatively and reductively metabolized to 2-chlorofatty acids and 2-chlorofatty alcohols [162]. Further metabolism of 2-chlorohexadonic acid affords 2-chloradipic acid (Fig. 8). These compounds are excellent biomarkers for hypochlorous acid because they retain the chlorine atom, and are produced by neutrophils [120,163] and monocytes [120] *in vivo* by a myeloperoxidase-dependent process. They are also amenable to quantitative and sensitive detection using mass spectrometry. They have been detected in atheroma [164] and in LDL from atherosclerotic lesions [165], while 2-chloradipic acid is excreted in the urine of humans and rats [166]. 2-Chlorohexadecanal is detected by gas chromatography with mass spectrometry after derivatisation while its metabolites are measured using liquid chromatography with tandem mass spectrometry. An excellent recent review describes these methods in detail [167]. Quantitative analyses of these novel chlorinated lipids should complement the information gained from investigating chlorination of proteins and enhance the understanding of how oxidation of lipids contributes to leukocyte-mediated injury and disease. Detection of 2-chloroadipic acid has the added advantage that it is the only biomarker of hypochlorous that can be measured with assurance in urine.

7.3. Detection of glutathione sulfonamide

Glutathione sulfonamide (Fig. 8) is a stable product formed in good yields via a fast reaction of glutathione (Glu-Cys-Gly) with hypochlorous acid [138,139]. The sulfonamide linkage encompasses the sulfur atom of the cysteine residue and the amine moiety of the N-terminal glutamate residue. Linked together they form a nine membered cyclic structure [168]. Its formation is sufficiently selective for hypochlorous acid to make it a good biomarker. Hypobromous acid and peroxynitrite can form glutathione sulfonamide but in much lower yields [139]. Glutathione sulfonamide is produced when neutrophils and endothelial cells are treated with hypochlorous acid but in much lower yields than glutathione disulfide (GSSG) [169]. Clinical studies looking for the formation of glutathione sulfonamide have focused on respiratory diseases because the concentration of glutathione in epithelial lining fluid is relatively high at about 400 μM [170]. Hence, glutathione in the airways is a likely target for any hypochlorous acid produced by stimulated

neutrophils. Indeed, glutathione sulfonamide was readily detected in the lung lavage fluid from pre-term babies with respiratory infections [171]. In these samples, its concentration correlated with levels of both myeloperoxidase and 3-chlorotyrosine in proteins. These results supported its use as a biomarker of hypochlorous acid. Glutathione sulfonamide showed better sensitivity and selectivity for detecting bacterial growth than the either myeloperoxidase or 3-chlorotyrosine. Consequently, it has potential for detecting lung infections. In support of this claim, glutathione sulfonamide is also produced in the airways of children with cystic fibrosis [171]. Its levels are elevated in those children with respiratory infections compared to those without infections (unpublished result).

Glutathione sulfonamide is detected using liquid chromatography with stable isotope dilution tandem mass spectrometry [169]. The method is sensitive and quantitative with a limit of quantitation of 0.1 pmol. Immediately upon collection, samples are treated with N-ethylmaleimide to block the unreacted thiol group on glutathione to form an adduct (GS-NEM). Before analysis stable heavy isotopes of glutathione sulfonamide, GSSG and GS-NEM are added to the sample and proteins are precipitated. The analytes and their respective isotopes are then separated by reversed phase liquid chromatography and detected by selected reaction monitoring. The heavy isotopes are essential for accurate quantification as they account for losses during sample handling and ion suppression during analysis in the mass spectrometer. This method has potential to detect glutathione sulfonamide in plasma and urine. It should have wide applicability for demonstrating production of hypochlorous acid in inflammatory diseases. The method will also be useful for understanding the degree to which hypochlorous acid contributes to oxidation of glutathione in the airways.

8. Future prospects for biomarkers of hypochlorous acid

The continued pursuit of additional biomarkers for hypochlorous acid is likely to be rewarding. This is because more abundant products of the reactions of hypochlorous acid with biomolecules await discovery. These will include the specific oxidation products of cysteine and methionine residues on proteins. Thus, identification of sulfonamides within and between proteins should be fruitful as should the detection of dehydromethionine on the N-terminal of proteins. Indeed, we have found that the N-terminal methionine of calprotectin S100 A8 is converted to dehydromethionine and it is readily detected in bronchoalveolar lavage fluid from children with cystic fibrosis (Magon *et al.* unpublished). Development of specific antibodies to footprints of chlorine would be a huge advantage to show by immunohistochemistry where hypochlorous acid reacts in cells and tissue. Good results have been obtained with the HOP1 antibody to hypochlorous acid-modified proteins [172] but it also recognizes proteins oxidized by hypobromous acid [86]. Finally, further development of specific and sensitive reaction-based fluorescent probes should eventually illuminate the importance of hypochlorous acid in innate immunity and inflammation.

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