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Loss of 3-chlorotyrosine by inflammatory oxidants: Implications for the use of 3-chlorotyrosine as a bio-marker *in vivo*

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ABSTRACT

Activated neutrophils generate the potent oxidant hypochlorous acid (HOCl) from the enzyme myeloperoxidase (MPO). A proposed bio-marker for MPO-derived HOCl *in vivo* is 3-chlorotyrosine, elevated levels of which have been measured in several human inflammatory pathologies. However, it is unlikely that HOCl is produced as the sole oxidant at sites of chronic inflammation as other reactive species are also produced during the inflammatory response. The work presented shows that free and protein bound 3-chlorotyrosine is lost upon addition of the pro-inflammatory oxidants, HOCl, peroxynitrite, and acidified nitrite. Furthermore, incubation of 3-chlorotyrosine with activated RAW264.7 macrophages or neutrophil-like HL-60 cells resulted in significant loss of 3-chlorotyrosine. Therefore, at sites of chronic inflammation where there is concomitant $ONOO^-$ and HOCl formation, it is possible measurement of 3-chlorotyrosine may represent an underestimate of the true extent of tyrosine chlorination. This finding could account for some of the discrepancies reported between 3-chlorotyrosine levels in tissues in the literature.

Activated neutrophils secrete the enzyme myeloperoxidase (MPO) which uses H_2O_2 and chloride ions to catalyse the generation of the reactive chlorine species (RCS) hypochlorous acid (HOCl) (Eq. 1).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \quad (1)$$

Up to 80% of the H_2O_2 generated by activated neutrophils is used to form 20–400 μM HOCl an hour [1–5]. In this paper we use "hypochlorous acid" (pKa = 7.46) to refer to the approximately 50% ionised mixture of HOCl and OCl^- species at pH 7.4 [6]. HOCl oxidises many important biomolecules such as sulphydryl and thioether moieties, plasma membrane ATPases, collagen, ascorbate, proteins including α_1-antiproteinase, nucleotides, and DNA repair enzymes [reviewed in [7,8]] as well as chlorinating DNA bases [9,10] and the amino acid tyrosine to form a bio-marker specific for RCS, 3-chlorotyrosine [11,12]. Activated neutrophils have been shown *in vitro* to utilise MPO, Cl_2, and HOCl to chlorinate free and protein bound tyrosine residues [11–14]. Tyrosine chlorination by neutrophils can also occur from nitryl chloride (NO_2Cl), formed from the reaction of nitric oxide-derived nitrite and HOCl [14]. Isolated neutrophils activated with cytochalasin B/f-met-leu-phe (fMLP) were recently reported to generate 58 ± 7 μM 3-chlorotyrosine/10^6 cells [13].

3-Chlorotyrosine has been observed in a variety of human diseases. Using analytical techniques such as gas-chromatography–mass spectrometry (GC–MS) or high performance liquid chromatography (HPLC), increased levels of this bio-marker have been detected in patients with atherosclerosis [15] glomerularnephritis [16], cystic fibrosis [17,18], rheumatoid arthritis [19,20], asthma [21], and Alzheimer's disease [22]. Monoclonal antibodies to HOCl-modified protein and LDL have revealed increased HOCl-mediated cellular damage in the diseased human kidney (nephrosclerosis, glomerulosclerosis, and tubulointerstitial fibrosis) [16,23]. Furthermore, antibodies selective for 3-chlorotyrosine have revealed extensive tyrosine chlorination in the livers of bile duct ligated mice [24] suggesting a role for HOCl in human liver disease. Therefore, 3-chlorotyrosine is emerging as a promising bio-marker of human disease.

However, it is likely that during chronic inflammation 3-chlorotyrosine is exposed to inflammatory oxidants in addition to HOCl, such as peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), nitric oxide ($^{\cdot}NO$), hydroxyl radical ($^{\cdot}OH$) or nitrite $O_2^{\cdot -}$ under acidic

Abbreviations: BSA, bovine serum albumin; $CuCl_2$, copper (II) chloride; fMLP, f-met-leu-phe; HPLC, high performance liquid chromatography; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; ^-OCl, hypochlorite anion; HOCl, hypochlorous acid; IFN-γ, interferon gamma; LPS, lipopolysaccharide; $^{\cdot}NO$, nitric oxide; $O_2^{\cdot -}$, nitrite anion; HNO_2, nitrous acid; $ONOO^-$, peroxynitrite anion; RCS, reactive chlorine species; RNS, reactive nitrogen species; ROS, reactive oxygen species; $O_2^{\cdot -}$, superoxide anion.

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conditions. The stability of 3-chlorotyrosine in the presence of these additional physiologically relevant oxidants is unknown. We previously showed that 3-nitrotyrosine, a widely used biomarker for reactive nitrogen species (RNS) such as the pro-inflammatory oxidant peroxynitrite ($ONOO^-$), is rapidly and extensively depleted after addition of HOCl [25]. Therefore, it was pertinent to examine the fate of 3-chlorotyrosine under similar conditions and in the presence of activated, oxidant producing inflammatory cells.

Materials and methods

Materials. Bovine serum albumin (BSA), 3-chlorotyrosine, methionine, potassium dihydrogen phosphate (KH_2PO_4), dipotassium dihydrogen phosphate (K_2HPO_4), hydrogen peroxide (H_2O_2) solution, copper (II) chloride ($CuCl_2$), hypoxanthine, and xanthine oxidase (X-4500) were purchased from Sigma Chemical Company (Dorset, England). Sodium hypochlorite solution, ascorbic acid, and manganese dioxide (MnO_2) were purchased from Aldrich (Dorset, England) and HPLC grade methanol obtained from Romil Ltd. (Cambridgeshire, England). Cellu.-Sep dialysis membranes with a relative molecular mass cut off of 3500 were obtained from Pierce Chemical Co. (IL, USA). Spermine Nonoate was purchased from Alexis (San Diego, USA). Purified water used for all solutions was obtained from ELGA Water Purification Systems, (Buckinghamshire, England), Maxim unit.

Measurement of peroxynitrite, hypochlorous acid, and hydrogen peroxide. Synthesis of hydrogen peroxide-free peroxynitrite was essentially as described in Ref [26] and quantified before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1} [27]. Hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2) concentration were quantified spectrophotometrically (molar absorption coefficients at 290 nm (pH 12, $\varepsilon = 350\ M^{-1}\ cm^{-1}$ [6] and 240 nm $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$, respectively) immediately before use.

Loss of free 3-chlorotyrosine. A 1 mM stock concentration of 3-chlorotyrosine was prepared by dissolving the desired amount in phosphate buffer (250 mM K_2HPO_4–KH_2PO_4, pH 7.4). Various concentrations of $ONOO^-$, HOCl or H_2O_2 were then added and the samples incubated for a 1 h at 37 °C. To generate $H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$, 0.25 U/ml xanthine oxidase and hypoxanthine (1 mM) was used and samples incubated at 37 °C for 1 h [25]. To generate ·OH, 3-chlorotyrosine was incubated with ascorbate (100 μM), $CuCl_2$ (100 μM), and H_2O_2 (1 mM) for 1 h at 37 °C [25]. To examine the effects of nitric oxide (· NO) on 3-chlorotyrosine, the ·NO-donor spermine nonoate was used at a final concentration of 1 mM for 1 h. The pH was measured after every experiment and found to be 7.4–7.45. Where time course studies were performed with HOCl, the reaction was quenched by addition of ice cold methionine (final concentration 1 mM) [9,10].

The effect of acidified nitrite (pH 3.4) on 3-chlorotyrosine was also examined. 3-Chlorotyrosine (100 μM) was incubated at 37 °C in phosphate buffer (250 mM KH_2PO_4–H_3PO_4, pH 3.4) for 15 min and $NaNO_2$ (dissolved in water) added for 1 h at 37 °C.

Exposure of BSA to HOCl: loss of protein 3-chlorotyosine by reactive species. Bovine serum albumin (BSA) was dissolved in phosphate buffer (250 mM K_2HPO_4–KH_2PO_4, pH 7.4) to a concentration of 10 mg/ml and exposed to 1 mM HOCl (final concentration) and incubated at 37 °C for 1 h. The samples were then dialysed against water for 24 h. After dialysis the concentration of protein remaining was measured using a BCA kit from Pierce (#23235) and the solution aliquoted into plastic test tubes containing phosphate buffer (250 mM K_2HPO_4–KH_2PO_4, pH 7.4) to give a final protein concentration of 4 mg/ml, incubated at 37 °C for 15 min then either HOCl, H_2O_2, or $·OH/NO_2^-$/·NO-generating systems added for 1 h at 37 °C. To examine the effect of acidified nitrite on protein bound 3-chlorotyrosine, chlorinated BSA was incubated at 37 °C in phosphate buffer (250 mM KH_2PO_4–H_3PO_4, pH 3.4) for 15 min, $NaNO_2$ added and the samples further incubated at 37 °C for 1 h. After oxidant treatment, samples were dialysed against water for 24 h and 1 mg total protein was freeze dried overnight. The protein solutions were then hydrolysed for 24 h in 6 M HBr containing 1% (v/v) phenol at 110 °C in evacuated glass hydrolysis tubes, followed by freeze drying overnight [25].

Cell culture. RAW264.7 macrophages and human promyelocytic HL-60 cells were obtained from the American Tissue Culture Collection (Gaithersberg, MA, USA) and cultured as described [28,29]. RAW264.7 cells were activated for 8 h in serum free media containing 1 μg/ml lipopolysaccharide (LPS) and interferon-γ (IFN-γ; 10 U/ml) to induce nitric oxide, superoxide, and peroxynitrite synthesis [28]. Cells (5×10^6/ml) were then incubated in warm (37 °C) phenol-red free Earle's Balanced Salt Solution (EBSS) containing 100 μM 3-chlorotyrosine and residual 3-chlorotyrosine measured as described below. HL-60 cells were differentiated into neutrophilic HL-60 cells with DMSO (1.3%; 7 days) [29] prior to use and activated in warm (37 °C) phenol-red free EBSS containing 100 μM 3-chlorotyrosine and a solution of phorbol myristate acetate (PMA; 5 μM)/fMLP (1 μM), added to induce respiratory burst and HOCl synthesis, as described in [29].

Measurement of 3-chlorotyrosine by high performance liquid chromatography (HPLC). HPLC analysis was performed using an Agilent 1100 series HPLC machine with 3-chlorotyrosine detection achieved using UV-photodiode array (Agilent model #G1315B) in series with an electrochemical detector set at 1.0 V (Agilent model #1049A). Immediately prior to HPLC analysis, samples were dissolved in 1.0 ml of mobile phase buffer (250 mM K_2HPO_4–KH_2PO_4, pH 3.01) containing 6% (v/v) methanol. Measurement of 3-chlorotyrosine was performed using a Spherisorb 5 μm ODS2 C_{18} column (HPLC Technology Ltd., Cheshire, England) (v/v) at a flow rate of 1.0 ml/min through a Agilent 1100 series isocratic pump (model #G1311A). The identity of 3-chlorotyrosine was confirmed by retention time, spiking with standards and examining the absorbance spectrum (photo-diode array). Peak area was measured and concentrations calculated from a standard curve. The retention for 3-chlorotyrosine under these conditions was 6.86 min, detection limit 1.0 μM.

Data analysis. Data are expressed as means ± standard deviation of the mean (SD) of separate experiments ($n \geqslant 6$). For significance testing, ANOVA was used ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) and concentration-dependent effects investigated with post hoc Dunnett's test using SPSS 12.0 software.

Results

Loss of 3-chlorotyosine by reactive oxygen species

Fig. 1A shows the effect of various reactive species on free 3-chlorotyrosine added for 1 h. Small but significant loss of 3-chlorotyrosine was observed with acidified nitrite (1 mM; $p < 0.1$, Fig. 1A). In contrast, pre-incubation of 3-chlorotyrosine with either $NaNO_2$ at pH 7.4, H_2O_2 or ·OH, O_2·$^-$, and ·NO-generating systems or freshly decomposed $ONOO^-$ [26] for 1 h did not result in its significant loss. However, the addition of increasing concentrations of HOCl led to extensive and concentration-dependent loss of 3-chlorotyrosine with significant loss achieved using 60 μM HOCl (68.3 ± 2.6% 3-chlorotyrosine remaining compared to untreated control; $p < 0.01$) (Fig. 1B). Higher concentrations of $ONOO^-$ (125 μM) caused similar loss (65.1 ± 4.2% 3-chlorotyrosine remaining compared to untreated control; $p < 0.01$; Fig. 1B).

The reaction of HOCl with 3-chlorotyrosine was rapid and substantial loss achieved after only 10 min with 60 μM HOCl ($p < 0.05$; Fig. 1C). Further loss was observed with increasing length of incubation. No additional peaks were detected by HPLC under any of our reaction conditions.

Loss of protein 3-chlorotyrosine by reactive species

Exposure of BSA to HOCl leads to chlorination of some of the tyrosine residues of the protein, to an extent that is broadly dependent on to the concentration of HOCl added. A concentration of 1 mM HOCl was added to stock BSA (20 mg/ml; 60.6 μM) and the resultant stock chlorinated BSA used for further studies. This stock solution contained 25.5 nmol 3-chlorotyrosine/mg protein (0.42 μM 3-chlorotyrosine/μM BSA). The addition of increasing concentrations of $ONOO^-$ to chlorinated BSA gave substantial and significant concentration-dependent loss of chlorinated tyrosine residues in the protein (Fig. 2A). Higher concentrations of $ONOO^-$ were required to cause similar loss of protein bound 3-chlorotyrosine levels compared to the free amino acid (Fig. 1) presumably due to competing reactions of other amino acids with $ONOO^-$. The addition of 60 μM $ONOO^-$ was sufficient to give significant loss of 3-chlorotyrosine (62.71 ± 3.0% 3-chlorotyrosine remaining; $p < 0.001$) compared to untreated controls. In contrast neither acidified nitrite (pH 3.4), H_2O_2 or ·OH-, ·NO-, and O_2·$^-$ -generating systems significantly depleted protein 3-chlorotyrosine (Fig. 2B).

Loss of 3-chlorotyrosine by activated inflammatory cells

The addition of 3-chlorotyrosine (100 μM) to vehicle treated RAW264.7 or HL-60 cells for up to 1 h did not result in the significant uptake of 3-chlorotyrosine from the culture media (Fig. 3). However, when 3-chlorotyrosine was added for 1 h to IFN-γ/LPS -activated RAW264.7 macrophages or PMA/fMLP-activated differentiated HL-60 cells, significant ($p < 0.01$) 3-chlorotyrosine was lost (Fig. 3).

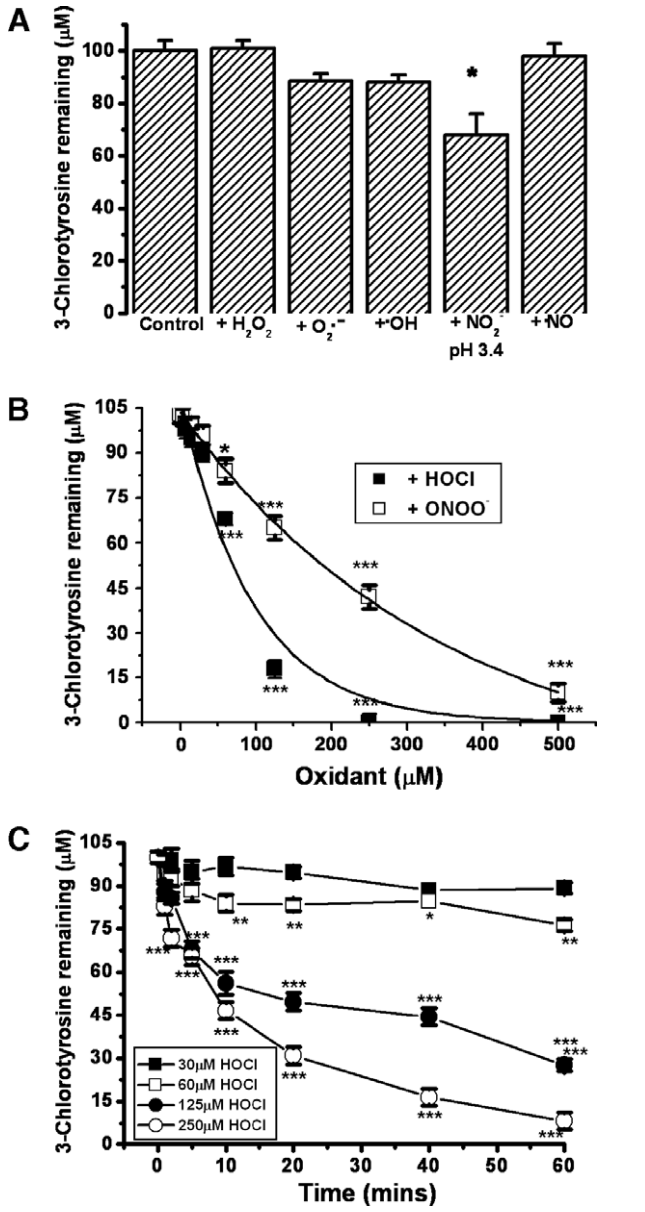

Fig. 1. Loss of free 3-chlorotyrosine by HOCl and $ONOO^-$ but not by other reactive species. (A) Effect of various reactive species on 3-chlorotyrosine. (B) Concentration-dependent loss of 3-chlorotyrosine by $ONOO^-$ and HOCl. (C) Time dependent loss of 3-chlorotyrosine by HOCl. 3-Chlorotyrosine (100 μM) in 250 mM phosphate buffer, pH 7.4, was incubated at 37 °C and increasing concentrations of either HOCl, $ONOO^-$, H_2O_2 or $\cdot OH/NO_2^-/\cdot NO$ generating systems for 1 h. Where HOCl was used, the reaction was quenched with the addition of ice cold methionine to a final concentration 1 mM. Residual 3-chlorotyrosine was measured by HPLC as described in Materials and methods. Data are expressed as Means ± SD of six separate experiments. $^{***}p < 0.01$ compared to 3-chlorotyrosine without oxidant addition.

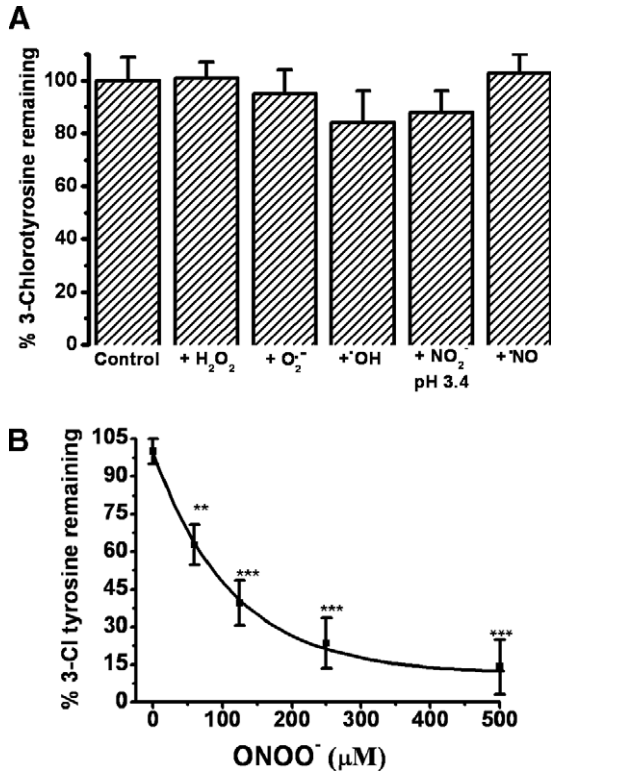

Fig. 2. Loss of protein 3-chlorotyrosine by $ONOO^-$ but not by other reactive species. (A) Effect of various reactive species on protein bound 3-chlorotyrosine. (B) Concentration-dependent loss of 3-chlorotyrosine by $ONOO^-$. Chlorinated BSA in 250 mM phosphate buffer, pH 7.4, was incubated at 37 °C with either $ONOO^-$, HNO_2, H_2O_2 or $\cdot NO/\cdot OH/O_2\cdot^-$ generating systems for 1 h. Residual 3-chlorotyrosine was measured by HPLC as described in Materials and methods. Data are expressed as Means ± SD of six separate experiments. $^{***}p < 0.01$ compared to chlorinated BSA without oxidant addition.

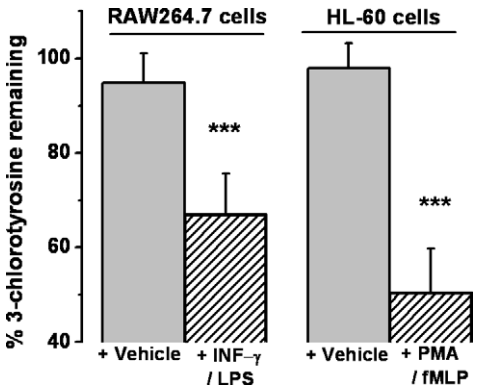

Fig. 3. Loss of 3-chlorotyrosine by activated inflammatory cells. (A) Effect of untreated and IFN-γ (10 U/ml)/LPS (1 μg/ml)-activated RAW264.7 macrophages. RAW264.7 cells were activated for 18 h to induce RNS and ROS formation. After washing with warm (37 °C) EBSS, cells were incubated in EBSS containing fresh IFN-γ/LPS (or vehicle) for 2 h at 37 °C in a cell culture incubator and 100 μM 3-chlorotyrosine added. (B) Effect of untreated and PMA/fMLP-activated HL-60 cells. After washing with warm (37 °C) EBSS, cells were incubated in EBSS containing fMLP (1 μM) and PMA (5 μM) (or vehicle) and 100 μM 3-chlorotyrosine added. After 1 h, cells were then pelleted by centrifugation and residual 3-chorotyrosine measured in the supernatant by HPLC as described in Materials and methods. Data are expressed as Means ± SD of six separate experiments. $^{***}p < 0.01$ compared to 3-chlorotyrosine added to non-activated cells.

Discussion

Hypochlorous acid (HOCl) formation has long been associated with chronic inflammation [1–5]. However, only recently has there been an interest in the identification of specific bio-markers for HOCl and RCS *in vivo*. 3-Chlorotyrosine has been suggested to fulfil this role [11,12]. The generation of antibodies to recognise HOCl-modifed protein [16] and those specific for 3-chlorotyrosine [24] as well as the development of robust and highly sensitive analytical techniques [11–13,15–17,22] to measure 3-chlorotyrosine *in vivo* has revealed extensive HOCl-mediated chlorination in a growing list of human diseases [15–24]. However, the extent of 3-chlorotyrosine formation *in vivo* could be influenced by metabolism [30] inflammatory oxidants other than the oxidant responsible for its formation (HOCl), as has been observed for the RNS bio-marker 3-nitrotyrosine [25]. For example H_2O_2, $O_2\cdot^-$, $\cdot NO$, $\cdot OH$, and $ONOO^-$ are also produced during inflammation. H_2O_2

and ·OH as well as HOCl induce protein oxidation *in vivo* [31]. 3-Chlorotyrosine may also be exposed to RNS since extensive tyrosine nitration (3-nitrotyrosine), as a bio-marker for RNS such as $ONOO^-$ and HNO_2 [32,33] is observed as either the free amino acid or protein bound in plasma, tissues or cells from patients with atherosclerosis [34,35], asthma [36], Alzheimer's disease [37,38], liver injury [39]; (including animal models of bile duct ligation [39]) and rheumatoid arthritis [40,41] i.e. the same pathologies described as involving 3-chlorotyrosine formation [15–24].

In our study, both free and protein bound 3-chlorotyrosine were shown to be lost upon addition of low concentrations of $ONOO^-$, high concentrations of acidified NO_2^- and activated inflammatory cells *in vitro* (Fig. 3), but not H_2O_2, or ·OH-, ·NO-, and $O_2^{\cdot -}$-generating systems (Figs. 1 and 2). Interestingly, free 3-chlorotyrosine was also depleted by the neutrophil-derived oxidant responsible for its formation *in vivo*, HOCl and by MPO containing activated neutrophilic HL-60 cells (Fig. 3). Therefore, our *in vitro* findings suggest that one must be cautious when using 3-chlorotyrosine formation as a quantitative index of the formation of HOCl or of RCS generally at inflammatory sites. Levels of 3-chlorotyrosine measured under inflammatory conditions could lead to an underestimation of the true extent of protein chlorination. Our laboratory is currently attempting to identify the product(s) formed from the loss of 3-chlorotyrosine by HOCl and $ONOO^-$ since they may serve as additional bio-markers of RCS and RNS formation *in vivo*.

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