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Loss of 3-Nitrotyrosine on Exposure to Hypochlorous Acid: Implications for the Use of 3-Nitrotyrosine as a Bio-marker *in Vivo*

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Peroxynitrite ($ONOO^-$) is a reactive nitrogen species which *in vivo* is often assessed by the measurement of free or protein bound 3-nitrotyrosine. Indeed, 3-nitrotyrosine has been detected in many human diseases. However, at sites of inflammation there is also production of the powerful oxidant hypochlorous acid (HOCl) formed by the enzyme myeloperoxidase. Low concentrations of HOCl (<30 μM) caused significant and rapid loss (<10 minutes) of free and protein bound 3-nitrotyrosine. In contrast, no loss of 3-nitrotyrosine was observed with hydrogen peroxide, hydroxyl radical, or superoxide generating systems. Therefore, under conditions where there is concomitant peroxynitrite and hypochlorous acid formation, such as at sites of chronic inflammation, it is possible that HOCl removes 3-nitrotyrosine. This may have implications when assessing the role of reactive nitrogen species in disease conditions and could account for some of the discrepancies reported between 3-nitrotyrosine levels in tissues.

The interaction of nitrogen monoxide ($NO^\bullet$) and superoxide ($O_2^{\bullet -}$) forms the cytotoxic product peroxynitrite (1), $ONOO^-$ (Equation 1).

$$NO^\bullet + O_2^{\bullet -} \rightarrow ONOO^- \qquad [1]$$

The rate constant for this formation has been determined to be 6.7 ($\pm$ 0.9) $\times$ 10^9 M^{-1} sec^{-1} (2). Under physiological conditions $ONOO^-$ has a half life of under 1 second and it is converted to its protonated form peroxynitrous acid, ONOOH, which in turn decays to generate multiple toxic products with the reactivities of the nitryl cation (NO_2^+), nitrogen dioxide radical ($^\bullet NO_2$) and hydroxyl radical ($^\bullet OH$). Peroxynitrite and species derived from it can oxidise lipids (3), proteins (4) and DNA (5,6). The addition of $ONOO^-$ to biological fluids leads to the depletion of ascorbate and thiols (7). One product often used as a marker of $ONOO^-$ production, 3-nitrotyrosine (3-NO_2 tyr), has been detected in various diseases and animal models of pathological conditions including rheumatoid arthritis, endotoxic shock, inflammatory bowel disease, atherosclerosis, acute lung injury, ageing of skeletal muscle, motor neurone disease and Alzheimer's disease (reviewed in (8)).

Activated neutrophils secrete the enzyme myeloperoxidase (MPO) which uses H_2O_2 and chloride ions to generate the powerful anti-bacterial agent hypochlorous acid (HOCl) (Equation 2) (9).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + HCl \qquad [2]$$

HOCl can oxidise many important biological molecules such as sulphydryl and thioether moieties (10,11), plasma membrane ATPases, collagen, ascorbate, proteins including α_1-antiproteinase, nucleotides, and DNA repair enzymes (12,13). It is also capable of chlorinating cholesterol and fatty acids in cell membranes (14), and DNA bases (15,16). HOCl has also been shown to chlorinate protein tyrosine residues to give 3-Cl tyrosine (3-Cl tyr) *in vitro* (17,18) and 3-Cl tyr has been detected in some atherosclerotic tissues (19). Hence, 3-NO_2 tyr and 3-Cl tyr have both been proposed as bio-markers of $ONOO^-$ and HOCl formation at sites of inflammation and their levels are sometimes taken

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Abbreviations used: HOCl, hypochlorous acid; ^-OCl, hypochlorite anion; H_2O_2, hydrogen peroxide; $^\bullet OH$, hydroxyl radical; $O_2^{\bullet -}$, superoxide anion; 3-NO_2 tyr, 3-nitrotyrosine; NANTE, *N*-acetyl-3-nitrotyrosine ethyl ester; 3-Cl tyr; 3-chlorotyrosine; BSA, bovine serum albumin; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; $NO^\bullet$, nitrogen monoxide; NO_2^-, nitrite anion; RNS, reactive nitrogen species; RCS, reactive chlorine species; ROS, reactive oxygen species; $CuCl_2$, copper (II) chloride; HPLC, high performance liquid chromatography.

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as an indication of the amount of $ONOO^-$ and HOCl formed. However, in this paper, we show that 3-nitrotyrosine is depleted on addition of HOCl and hence one must be cautious when using 3-nitrotyrosine formation as a quantitative index of the formation of $ONOO^-$ (8) or of reactive nitrogen species generally (20) at sites of inflammation.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), 3-nitrotyrosine (3-NO_2 tyr), N-acetyl-3-nitrotyrosine ethyl ester, 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. (Illinois, USA). Purified water used for all solutions was obtained from ELGA Water Purification Systems, (Buckinghamshire, England), Maxim unit.

Synthesis of peroxynitrite. Synthesis of peroxynitrite was essentially as described in ref (1). Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for one second and the reaction quenched with ice cold NaOH (1.2 M). Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (−20°C) and the top layer of the solution collected for the experiment. Concentrations of stock $ONOO^-$ were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1} (21). Concentrations of 200-250 mM were usually obtained. Once thawed, $ONOO^-$ solutions were kept on ice for no longer than 30 minutes before use.

Measurement of HOCl and H_2O_2. Hypochlorite (OCl^-) and hydrogen peroxide (H_2O_2) concentration were quantified spectrophotometrically (molar absorption coefficients at 290 nm (pH 12, $\epsilon = 350$ M^{-1} cm^{-1} (22) and 240 nm $\epsilon = 43.6$ M^{-1} cm^{-1} respectively) immediately before use. Hypochlorous acid (HOCl) has a pKa of 7.46 (22), thus the term HOCl is used here to represent a mixture of HOCl and OCl^-.

Loss of free 3-NO_2 tyrosine. A 1mM stock concentration of 3-NO_2 tyrosine was prepared by dissolving the desired amount in phosphate buffer (250 mM K_2HPO_4-KH_2PO_4, pH 7.4). In a plastic test tube 100 μl of tyrosine solution was added to 900 μl phosphate buffer pH 7.4 and the solution incubated in a water bath at 37°C for 15 minutes. After this time various concentrations of $ONOO^-$, HOCl or H_2O_2 were added and the tubes vortexed for 10 seconds and incubated for a further 1 hour at 37°C. To generate $O_2^{\cdot-}$, 0.25 units/ml xanthine oxidase was incubated at 37°C in phosphate buffer (250 mM K_2HPO_4-KH_2PO_4, pH 7.4) in a reaction volume of 2.0 mls with increasing concentrations of hypoxanthine for 1 hour. Ascorbate (100 μM) and $CuCl_2$ (100 μM) in the presence of increasing concentrations of H_2O_2 at 37°C in phosphate buffer (250 mM K_2HPO_4-KH_2PO_4, pH 7.4 total reaction volume of 2.0 mls) was used to generate $^{\cdot}OH$. 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).

Exposure of BSA to $ONOO^-$: Loss of 3-NO_2 tyr by reactive oxygen 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 10 mM $ONOO^-$ (final concentration) and incubated in a water bath at 37°C for 1 hour. The samples were then dialysed against water for 24 hours to remove any nitrate/nitrite from the decomposition of $ONOO^-$. After dialysis the concentration of protein remaining was measured (23) and the solution aliquotted 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 minutes and either HOCl, H_2O_2 or $^{\cdot}OH/O_2^{\cdot-}$ generating systems added for 1 hour at 37°C. Where time course studies with HOCl were performed the reaction was quenched with the addition of ice cold methionine (final concentration 1 mM). Samples were then dialysed against water for 24 hours and the protein concentration measured (23). A volume containing 1 mg total protein was added to glass vials and the samples freeze dried overnight. The protein solutions were then hydrolysed for 24 hours in 6 M HBr containing 1% (v/v) phenol at 110°C in evacuated glass hydrolysis tubes, followed by overnight lyophilisation.

Measurement of 3-NO_2 tyrosine by high performance liquid chromatography (HPLC). Immediately prior to HPLC analysis, samples were dissolved in 1.0 ml of phosphate buffer (250 mM K_2HPO_4-KH_2PO_4, pH 3.01) containing 6% (v/v) methanol. Measurement of 3-NO_2 tyr was performed using a Spherisorb 5 μm ODS2 C_{18} column (HPLC Technology Ltd, Wellington House, Cheshire, England) with a guard column (Hibar from BDH, Poole, Dorset, England) and C_{18} cartridge as described previously (24). The eluent was 250 mM K_2HPO_4-$KHPO_4$, pH 3.01, with 6% methanol (v/v) at a flow rate of 1.0 ml/min through a Gynkotek isocratic pump (model 480) and UV-photodiode array (Gynkotek, model UVD-340, Cheshire, England) in series with an electrochemical detector (INTRO from Antec containing a glassy carbon working electrode and an Ag/AgCl electrode supplied by Presearch Ltd., Hertfordshire, England) set at 1.0 volt (24). The identity of 3-NO_2 tyr 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 detection limit for 3-NO_2 tyrosine (retention time of 16.2 mins) was 0.1 μM.

Data analysis. All graphs are plotted with mean ± standard error of the mean (sem). In all cases the mean values were calculated from data taken from at least 3 separate experiments performed on separate days using freshly prepared reagents. Where significance testing was performed, an independent t-test (Students; 2 populations) was used; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RESULTS

Loss of 3-NO_2 tyrosine by reactive oxygen. Figure 1A shows the effect of various reactive species on free 3-NO_2 tyr. The addition of increasing concentrations of HOCl led to extensive and concentration-dependent loss of 3-NO_2 tyr with significant loss (83.36 ± 0.92% 3-NO_2 tyr remaining compared to untreated control) achieved using 15 μM HOCl. The characteristic yellow colour of 3-NO_2 tyr was also lost. In contrast, no significant loss of 3-NO_2 tyr was observed with either freshly decomposed $ONOO^-$ (1) or with 1 mM $ONOO^-$. The reaction of HOCl with 3-NO_2 tyr was rapid and substantial loss achieved after only 5 minutes with low concentrations of HOCl (30 μM; Fig. 1B). Depletion of 3-NO_2 tyr by HOCl was essentially independent of pH (pH 5, 7.4 and 9.0 tested; data not shown).

The addition of HOCl to 3-NO_2 tyr did not result in the formation of any detectable 3-Cl tyr (sensitivity limit, 0.1 μM) and no additional peaks were detected by HPLC under any of our reaction conditions. H_2O_2 or

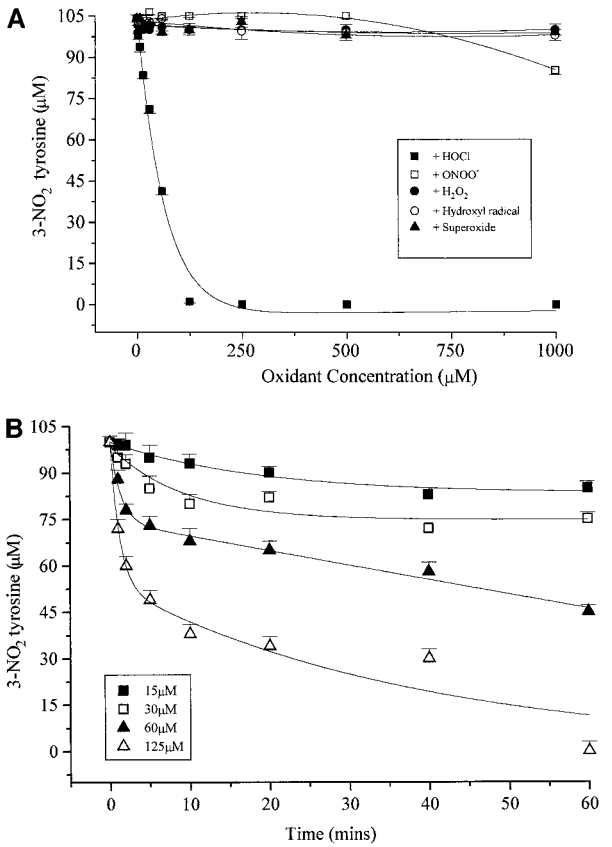

FIG. 1. Loss of free 3-NO_2 tyr by reactive chlorine species but not by other reactive species. (A) Concentration dependent loss of 3-NO_2 tyr by various reactive species. (B) Time dependent loss of 3-NO_2 tyr by HOCl. 3-NO_2 tyr (100 μM) in 250 mM phosphate buffer, pH 7.4, was incubated at 37°C with increasing concentrations of HOCl, $ONOO^-$, H_2O_2, or $^{\bullet}OH/O_2^{\bullet -}$ generating 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-NO_2 tyr was measured by HPLC as described under Materials and Methods. Data are expressed as means ± sem of 4 or more separate experiments.

systems generating $^{\bullet}OH$ ($CuCl_2$/ascorbate/H_2O_2) or $O_2^{\bullet -}$ (hypoxanthine/xanthine oxidase) did not significantly deplete 3-NO_2 tyr levels (Fig. 1A).

3-NO_2 tyr loss was also followed spectrophotometrically (Fig. 2A). Addition of increasing amounts of HOCl to 3-NO_2 tyr led to a loss in absorbance at 425 nm, 275 nm and 225 nm (Fig. 2A). Incubation of HOCl with N-acetyl-3-nitrotyrosine ethyl ester (NANTE) also led to a diminution of the peaks at 425 nm, 275 nm and 225 nm (Fig. 2B).

Loss of protein 3-NO_2 tyrosine by reactive species. Exposure of BSA to $ONOO^-$ leads to nitration of some of the tyrosine residues of the protein, to an extent that is broadly dependent on to the concentration of $ONOO^-$ added. A concentration of 10 mM $ONOO^-$ was added to stock BSA (20 mg/ml; 60.6 μM) and the resultant stock nitrated BSA used for further studies. This stock solution contained 8 nmoles 3-NO_2 tyr/mg protein (0.13 μM 3-NO_2 tyr/μM BSA). The addition of increasing concentrations of HOCl to nitrated BSA gave substantial concentration-dependent loss of nitrated tyrosine residues in the protein (Fig. 3A). The addition of 60 μM HOCl was sufficient to give significant loss of 3-NO_2 tyr (78.0 ± 3.0% 3-nitrotyrosine remaining) compared to untreated controls. The reaction of HOCl with protein bound 3-NO_2 tyr was dependent on the time of exposure. After only a 5 minute exposure to HOCl (500 μM), significant loss of protein bound 3-NO_2 tyr was observed. Further loss was observed with increasing length of incubation. This loss was not significantly affected by the pH of the reaction mixture (pH 5.0, pH 7.4 and pH 9.0 used; data not shown). H_2O_2 or $^{\bullet}OH$ and $O_2^{\bullet -}$ generating systems in contrast, did not deplete protein bound 3-NO_2 tyr.

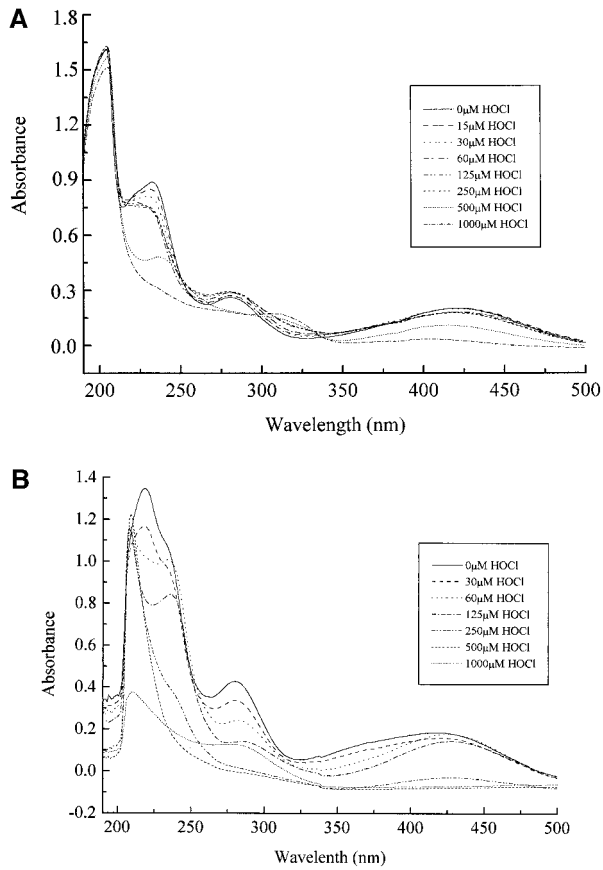

FIG. 2. Loss of 3-NO_2 tyr by HOCl: Spectrophotometric measurements. (A) Concentration dependent depletion of 3-NO_2 tyr by HOCl. 3-NO_2 tyr (100 μM) was incubated at pH 7.4 for 1 h at 37°C in the presence of increasing concentrations of HOCl. (B) Loss of *N*-acetyl 3-nitrotyrosine ethyl ester (NANTE) by HOCl. NANTE (100 μM) was incubated at 37°C, pH 7.4, for 1 h with increasing concentrations of HOCl. Experiments were conducted as described under Materials and Methods. Results are representative of 3 or more separate experiments. The UV spectrophotometer was blanked with phosphate buffer and experiments conducted as described under Materials and Methods.

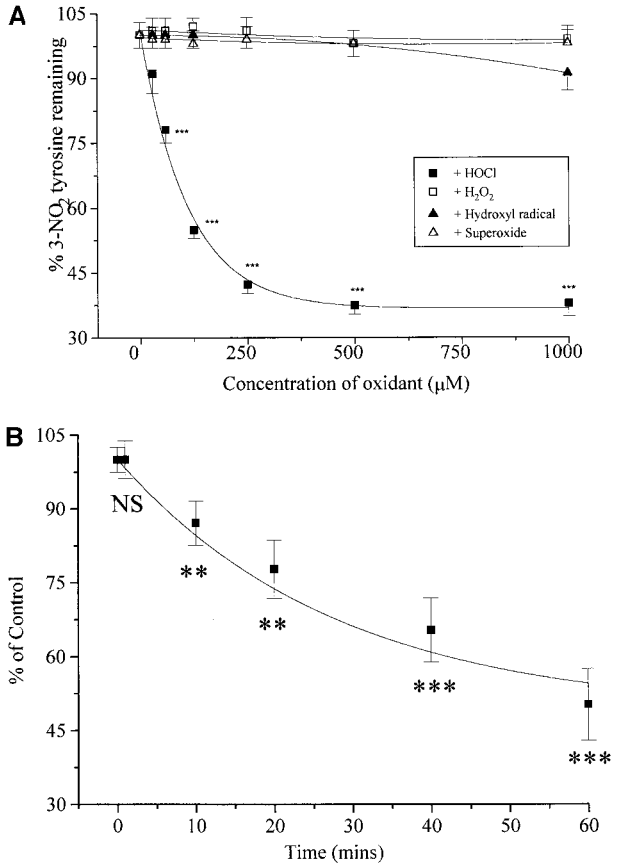

FIG. 3. Loss of protein bound 3-NO_2 tyr by reactive chlorine species but not by other reactive species (A) Concentration dependent loss of 3-NO_2 tyr by various reactive species. (B) Time dependent loss of protein bound 3-NO_2 tyr by HOCl. Nitrated BSA in 250 mM phosphate buffer, pH 7.4, was incubated at 37°C with increasing concentrations of HOCl, $ONOO^-$, H_2O_2, or $^{\cdot}OH/O_2^{\cdot-}$ generating systems for 1 h. Where HOCl was used, the reaction was quenched by addition of ice cold methionine to a final concentration of 1 mM. Residual 3-NO_2 tyr was measured by HPLC as described under Materials and Methods. Data are expressed as means ± sem of 3 or more separate experiments.

DISCUSSION

The generation of $ONOO^-$ *in vivo* has been implicated in many disease conditions and its production is thought to contribute to the overall disease pathology by interfering with signal transduction mechanisms involving phosphorylation/dephosphorylation (25) adenylation (26) and protein assembly (27).

Both free and protein bound 3-NO_2 tyr are found *in vivo*. For example, 3-NO_2 tyr in the free form is found in rheumatoid arthritis, amyotrophic lateral sclerosis and protein bound 3-NO_2 tyr is found in acute lung injury, atherosclerosis, amyotrophic lateral sclerosis, Parkinson's disease and myocardial inflammation (reviewed in (8)). Therefore it was pertinent to examine the effects of other ROS on both free and protein bound 3-NO_2 tyr. Both forms of 3-NO_2 tyr were shown to be attacked by HOCl in a concentration and time dependent manner (Figs. 1-3). Low concentrations of HOCl (30 μM) were sufficient to substantially deplete free 3-NO_2 tyr and 60 μM sufficient for the protein bound 3-NO_2 tyr. The essentially pH independent nature of this loss suggests that both HOCl and its conjugate base (OCl^-) were capable of reacting with 3-NO_2 tyr.

N-acetyl-3-NO_2 tyrosine ethyl ester (NANTE) was used for spectral studies. This compound has the N-amino group blocked by an acetyl group and the carboxyl group blocked by an ethyl ester. Therefore, it is less likely to form chloramines than 3-NO_2 tyr. However, NANTE was rapidly depleted by HOCl. Accompanying this loss was the time and concentration dependent loss of absorbance at 425 nm, the area of the spectrum relating to the nitro group and a sharp loss in absorbance at 220 nm and 275 nm, the areas relating to the aromatic ring of NANTE (Figs. 3a and b). There was no detectable 3-Cl tyr or hydroxylated tyrosine residues in any of the samples, ruling out a simple substitution reaction (which is unlikely in any case). No extra peaks were observed when the reaction mixtures were analysed by HPLC. Attack on the aromatic ring may produce ring-opened products which could account for the loss in absorbance at 220 nm and 425 nm and the lack of extra compounds detected by HPLC.

Atherosclerotic tissue has been reported to contain 3-NO_2 tyr by some groups (28-30) but not by others (31,32). It has also been reported to contain 3-chlorotyrosine (19). Hence one reason for the variation could be destruction of 3-nitrotyrosine by HOCl production. It is also possible that blood/tissues have other 3-NO_2 tyr removing processes. Indeed, injection of either free 3-NO_2 tyr (33) or $ONOO^-$-treated rat albumin (containing protein bound 3-NO_2 tyr) (34) into rats results in its rapid clearance from the circulation ($t^1/_2$=1.67 hours; and 5.8 hours respectively).

The results presented here indicate that HOCl and $ONOO^-$, when generated at sites of inflammation could deplete free or protein bound 3-NO_2 tyr formed from their respective actions on free or protein bound tyrosine residues. The situation is complex because HOCl, by reaction with nitrite (NO_2^-) can generate nitryl chloride (NO_2Cl) (35,36), a species capable of both chlorination and nitration, and so the availability of NO_2^- is yet another important variable. Consequently any assessment of 3-NO_2 tyr as a bio-marker may be an underestimate of the true extent of reactive nitrogen species activity at inflammatory sites. Our laboratory is currently attempting to identify the product(s) formed from the loss of 3-NO_2 tyr by HOCl since they may serve as additional bio-markers of $ONOO^-$ and HOCl production *in vivo*.

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