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Formation of Nitrating and Chlorinating Species by Reaction of Nitrite with Hypochlorous Acid

A NOVEL MECHANISM FOR NITRIC OXIDE-MEDIATED PROTEIN MODIFICATION*

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Detection of 3-nitrotyrosine has served as an *in vivo* marker for the production of the cytotoxic species peroxynitrite ($ONOO^-$). We show here that reaction of nitrite (NO_2^-), the autoxidation product of nitric oxide ($^{\bullet}NO$), with hypochlorous acid (HOCl) forms reactive intermediate species that are also capable of nitrating phenolic substrates such as tyrosine and 4-hydroxyphenylacetic acid, with maximum yields obtained at physiological pH. Monitoring the reaction of NO_2^- with HOCl by continuous flow photodiode array spectrophotometry indicates the formation of a transient species with spectral characteristics similar to those of nitryl chloride ($Cl\text{-}NO_2$). Reaction of synthetic $Cl\text{-}NO_2$ with *N*-acetyl-L-tyrosine results in the formation of 3-chlorotyrosine and 3-nitrotyrosine in ratios that are similar to those obtained by the NO_2^-/HOCl reaction (4:1). Tyrosine residues in bovine serum albumin are also nitrated and chlorinated by NO_2^-/HOCl and synthetic $Cl\text{-}NO_2$. The reaction of *N*-acetyl-L-tyrosine with NO_2^-/HOCl or authentic $Cl\text{-}NO_2$ also produces dityrosine, suggesting that free radical intermediates are involved in the reaction mechanism. Our data indicate that while chlorination reactions of $Cl\text{-}NO_2$ are mediated by direct electrophilic addition to the aromatic ring, a free radical mechanism appears to be operative in nitrations mediated by NO_2^-/HOCl or $Cl\text{-}NO_2$, probably involving the combination of nitrogen dioxide ($^{\bullet}NO_2$) and tyrosyl radical. We propose that NO_2^- reacts with HOCl by Cl^+ transfer to form both *cis*- and *trans*-chlorine nitrite (Cl-ONO) and $Cl\text{-}NO_2$ as intermediates that modify tyrosine by either direct reaction or after decomposition to reactive free and solvent-caged $Cl^{\bullet}$ and $^{\bullet}NO_2$ as reactive species. Formation of $Cl\text{-}NO_2$ and/or Cl-ONO *in vivo* may represent previously unrecognized mediators of inflammation-mediated protein modification and tissue injury, and offers an additional mechanism of tyrosine nitration independent of $ONOO^-$.

Nitrogen monoxide (nitric oxide, $^{\bullet}NO$)[1] is produced by a variety of cells through the activity of constitutive and inducible forms of nitric oxide synthase (1). $^{\bullet}NO$ is an important endogenous mediator in such diverse biochemical and physiological processes as neurotransmission, smooth muscle relaxation, platelet aggregation and adhesion, macrophage-mediated cytotoxicity, and learning and memory (2, 3). Although basal levels of free $^{\bullet}NO$ are normally quite low (nanomolar), local $^{\bullet}NO$ concentrations have been shown to increase to levels ranging from 4 to 30 μM under pathologic conditions (4, 5).

$^{\bullet}NO$ reacts at a near diffusion-controlled rate with superoxide ($O_2^{\bullet-}$) ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) (6) to form the cytotoxic species peroxynitrite ($ONOO^-$). The formation of $ONOO^-$ is thought to be responsible, at least in part, for the observed toxicity associated with $^{\bullet}NO$ (7, 8). At physiological pH the protonated form of $ONOO^-$, peroxynitrous acid (ONOOH) ($pK_a = 6.8$), is highly unstable and rapidly decomposes to nitrate (NO_3^-). ONOOH is thought to 1) react directly with biological molecules via a vibrationally excited intermediate (ONOOH*), 2) decompose by homolytic dissociation to form nitrogen dioxide ($^{\bullet}NO_2$) and the hydroxyl radical ($^{\bullet}OH$), or 3) by heterolytic dissociation to form the nitryl cation (nitronium ion, NO_2^+) (reviewed in Ref. 9). $ONOO^-$/ONOOH reacts with proteins, leading to the oxidation of cysteine, methionine, and tryptophan residues, and can induce protein carbonyl formation and nonspecific fragmentation (10–12). In addition, $ONOO^-$/ONOOH can react readily with phenolic compounds to form nitrated, hydroxylated, and dimerized products (13–17), and nitration of free tyrosine, or tyrosine in proteins, has served as a "marker" and "index" of $ONOO^-$ formation *in vivo*.

Based upon tyrosine nitration assays and the formation of "peroxynitrite-specific" luminescence, stimulated macrophages (18), neutrophils (19), and endothelial cells (20) have been proposed to form significant quantities of $ONOO^-$ *in vitro*. In fact, the detection of 3-nitrotyrosine (NO_2-Tyr) in a variety of pathologic conditions *in vivo*, such as inflammatory lung disease (21), atherosclerosis (22), and rheumatoid arthritis (23), has been attributed to $ONOO^-$ formation. However, in all of these cases direct proof for the production of $ONOO^-$ in biolog-

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[1] The abbreviations used are: $^{\bullet}NO$, nitric oxide; $O_2^{\bullet-}$, superoxide; HOCl, hypochlorous acid; NO_2^-, nitrite; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; NO_3^-, nitrate; NO_2-Tyr, 3-nitrotyrosine; Cl-Tyr, 3-chlorotyrosine; HPA, 4-hydroxyphenylacetic acid; NO_2-HPA, 3-nitro-4-hydroxyphenylacetic acid; Cl-HPA, 3-chloro-4-hydroxyphenylacetic acid; Cl-Phe, chlorophenylalanine; NAT, *N*-acetyl-L-tyrosine; NAP, *N*-acetyl-L-phenylalanine; MPA, 4-methoxyphenylacetic acid; $Cl\text{-}NO_2$, nitryl chloride; Cl-ONO, chlorine nitrite; ROS, reactive oxygen species; RNS, reactive nitrogen species; HPLC, high pressure liquid chromatography; PDA, photodiode array.

ical systems is lacking, even though its formation *in vivo* is favorably predicted (24).

Under inflammatory conditions, multiple well characterized reactive oxygen species (ROS) are produced from phagocytic cells (25). For instance, stimulated neutrophils and macrophages produce significant levels of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) as a result of the activation of the respiratory burst oxidase (26). In the case of neutrophils, some of the H_2O_2 that is produced under these conditions is converted to the strong oxidant hypochlorous acid (HOCl) by the action of myeloperoxidase as shown in Reaction 1.

$$H_2O_2 + H^+ + Cl^- \rightarrow HOCl + H_2O$$

REACTION 1

HOCl produced from activated human neutrophils has been shown to react with amines (taurine, lysine, and arginine) and tyrosine to form *N*-chloramines and 3-chlorotyrosine (Cl-Tyr), respectively (27, 28), where the latter has been proposed to serve as a selective marker of HOCl production *in vivo* (29).

In addition to ROS, macrophages (30) and neutrophils (19) can also simultaneously produce large fluxes of $^{\cdot}NO$ through the activation of inducible nitric oxide synthase; however, the ability of human neutrophils to produce $^{\cdot}NO$ is debated (31). Once formed, $^{\cdot}NO$ can react with several biological targets, primarily thought to involve heme-iron, hyperreactive sulfhydryls and protein radicals (32–34). $^{\cdot}NO$ can also react with O_2 in aqueous solution to produce nitrite (NO_2^-) via a complex mechanism thought to involve a variety of reactive nitrogen species (RNS) including $^{\cdot}NO_2$ and dinitrogen trioxide (N_2O_3) (35). In fact, NO_2^- has been used as a marker of $^{\cdot}NO$ production *in vitro* and *in vivo* and has been shown to reach concentrations of up to 4 μM in synovial fluid from patients with rheumatoid arthritis (36) and as high as 20 μM in human airway fluids (37). These RNS produced during an inflammatory response could theoretically react with a number of ROS to form various novel species. Indeed, the interaction of HOCl with $^{\cdot}NO$ or NO_2^- has been proposed to form species capable of nitrosylating and nitrating organic substrates (38, 39).

The present study was undertaken to examine the potential interactions of $^{\cdot}NO$-derived RNS with the inflammatory oxidant HOCl in an attempt to characterize more fully the various species that may be formed under complex physiological inflammatory conditions. Our results indicate that NO_2^- reacts with HOCl to form an intermediate species, postulated to be nitryl chloride (Cl-NO_2) and/or chlorine nitrite (Cl-ONO), that is capable of nitrating, chlorinating, and dimerizing phenolic compounds including tyrosine. We propose that the formation of Cl-NO_2 and/or Cl-ONO by this reaction represents a novel mechanism of inflammation-mediated biological damage, and offers an additional or alternative mechanism of tyrosine nitration independent of $ONOO^-$ formation.

EXPERIMENTAL PROCEDURES

Materials—DL-Phenylalanine, DL-tyrosine, *N*-acetyl-L-tyrosine (NAT), *N*-acetyl-L-phenylalanine (NAP), NO_2-Tyr, Cl-Tyr, 4-hydroxyphenylacetic acid (HPA), 3-nitro-4-hydroxyphenylacetic acid (NO_2-HPA), chlorophenylalanine isomers, sodium hypochlorite (NaOCl), sodium nitrite ($NaNO_2$), and bovine serum albumin (BSA; essentially fatty acid-free) were obtained from Sigma. 3-Chloro-4-hydroxyphenylacetic acid (Cl-HPA), 4-methoxyphenylacetic acid (MPA), and nitronium tetrafluoroborate (NO_2BF_4; 0.5 M solution in sulfolane) were from Aldrich. Nitric acid, sulfuric acid, and chlorosulfonic acid were obtained from Fisher Scientific (Pittsburgh, PA). Nitrogen monoxide (nitric oxide, $^{\cdot}NO$) (3,000 ppm in O_2-free N_2) was obtained from Scott-Marrin, Inc. (Riverside, CA). Dityrosine was synthesized by oxidation of L-tyrosine with horseradish peroxidase (type I; Sigma) and H_2O_2. Peroxynitrite ($ONOO^-$) was synthesized and quantified as described previously (40). HOCl concentrations were determined spectrophotometrically at 290 nm (pH 12, ϵ = 350 M^{-1} cm^{-1}). All buffer solutions were treated with Dowex-50 chelating resin to remove transition metals prior to experiments.

$^{\cdot}NO$ Experiments—$^{\cdot}NO$ (3,000 ppm in O_2-free N_2) was bubbled through continuously stirred 100-ml solutions of phosphate buffer (100 mM KH_2PO_4, pH 7.4) at a flow rate of 20 ml/min. Buffer solutions were purged with either air or purified N_2 for 30 min prior to $^{\cdot}NO$ exposure and were continuously sparged with either gas throughout the experiment to maintain an oxygenated or deoxygenated solution, respectively. At various time points, aliquots of the solution were withdrawn and purged briefly with N_2 to remove residual $^{\cdot}NO$, and NO_2^- production was determined spectrophotometrically using Griess reagent (1% sulfanylamide, 0.1% *N*-(1-naphthyl)ethylenediamine, and 2.5% $H3PO_4$) (41). In separate experiments, HPA (5 mM) was added to the buffer solutions prior to $^{\cdot}NO$ exposure. At various time points, 0.5-ml aliquots of the solution were withdrawn from the reaction mixture and immediately reacted with HOCl (1 mM). After 10 min, the reaction mixture was adjusted to pH 10–11 with 1 M NaOH. NO_2-HPA was measured spectrophotometrically at 430 nm; ϵ = 4400 M^{-1} cm^{-1} (16).

Nitration and Chlorination Reactions—Buffered solutions (100 mM KH_2PO_4) of HPA, NAT, NAP, or MPA (1–5 mM) were adjusted to the desired pH (5.0–8.5) with either 10% NaOH or 5% H_3PO_4 prior to experimentation. Where appropriate, NO_2^- was added at the desired concentration (0.1–3.0 mM) in 5-ml sample volumes. HOCl was then added to the solutions at 25 °C as a small drop (<20 μl) while continuously vortexing. Although reactions are nearly instantaneous, incubations were allowed to proceed for 10 min before reduced GSH was added at 1 mM concentration to scavenge any unreacted HOCl. In separate experiments $ONOO^-$ (in 1.2 M NaOH) or NO_2BF_4 (in sulfolane) were reacted with the various substrates in a similar manner. The products of the reactions involving HPA as substrate were analyzed directly by HPLC. Since nitrated, chlorinated, and dimerized *N*-acetylated derivatives of tyrosine were not available, reaction mixtures utilizing NAT as substrate were first hydrolyzed (see below) to liberate the free amino acids and their modified products prior to HPLC analysis. Reaction mixtures involving MPA were transferred to a quartz cuvette, and the absorbance spectrum was measured between 280 and 500 nm at pH 7.4.

Reactions of BSA with NO_2^-/HOCl—NO_2^- and HOCl (both 500 mM) were loaded into 1-ml syringes that were attached to an automated syringe pump. Teflon tubing from both syringes converged into a single tube and allowed reaction of the two components for a brief period (<1 s). The reaction effluent was allowed to drop approximately 6 cm immediately into 10-ml solutions of BSA (10 mg/ml) in 100 mM KH_2PO_4 (pH 7.4), which were continuously stirred. The volume of each drop was calibrated (33 μl), and final concentrations of oxidant exposure were calculated. In some cases, NO_2^- in one of the syringes was replaced with 100 mM KH_2PO_4 (pH 7.4) so as to allow exposure of BSA to HOCl alone under the same conditions. Following addition of the NO_2^-/HOCl mixture or HOCl alone, the solutions were stirred for 15 min and were then quenched by the addition of excess GSH. NO_2-Tyr, Cl-Tyr, and dityrosine formation in the samples were determined by HPLC following acid hydrolysis as described below.

Synthesis of Cl-NO_2—Caution: the reagents used and products formed in this synthesis are highly irritant and corrosive to the eyes, skin, and mucous membranes. All of the procedures involved in the synthesis of Cl-NO_2 must be performed in a fume hood to ensure proper ventilation, and appropriate eye and skin protection must be worn. At room temperature, Cl-NO_2 exists as a gas (boiling point, −15 °C) and presents a serious inhalation hazard if not handled properly. The procedure for the synthesis of Cl-NO_2 is essentially that previously described (42, 43) with slight modification. Sulfuric acid (61 g) was added dropwise to vigorously stirred nitric acid (50 g) at 0 °C in a 500-ml three-necked round bottom flask equipped with a dropping funnel. A cold finger receiving flask (150 ml) was attached to the reaction vessel by a short segment of Teflon tubing, and the flask was immersed in a cooling mixture of dry ice/acetone. After 10 min, chlorosulfonic acid (85 g) was slowly added dropwise via the attached funnel into the mixture of nitric and sulfuric acids over a 4-h period. During the entire synthesis procedure, a gentle flow of N_2 gas was delivered through the apparatus to enhance the evolution and collection of gaseous Cl-NO_2. It is important that the chlorosulfonic acid is added at a slow enough rate such that brown gas does not appear above the reaction mixture. The colorless gaseous Cl-NO_2 evolved from the reaction mixture was carried by the gentle flow of N_2 into the cold finger receiving flask, where it condensed as a pale yellow liquid. The product was purified by passing ozonized air through the liquefied gas to oxidize any nitrosyl chloride (Cl-NO) that may have been present as a contaminant. The product was carefully transferred to sealed glass vials and stored at −80 °C until

used in experiments. The yield of Cl-NO_2 is typically 80–90% (approximately 50 g), and the purity has been reported to be 98–99% (42). To confirm the identity of the reaction product, the purified product was diluted in methanol, and the absorbance spectrum was immediately measured. The observed absorbance spectrum of synthetic Cl-NO_2 showed a series of characteristic absorption maxima between 300 and 400 nm, similar to that reported previously (43).

Nitryl Chloride Exposures—Cl-NO_2 (10 ml) was placed in a 50-ml sparging flask immersed in a cooling dry ice/acetone bath. A stream of N_2 gas was allowed to flow through a glass tube fitted with a fritted glass fitting, which was submerged into the undiluted Cl-NO_2, and was bubbled through the liquid at a flow rate of 75 ml/min. Gaseous Cl-NO_2 evolved into the headspace exited through a glass tube connected to the top of the flask into a glass reaction vessel containing a solution of the analyte to be exposed. Cl-NO_2 in N_2 gas was allowed to bubble through the 100 mM phosphate-buffered solutions (25 ml, pH 7.4) of NAT (5 mM), NAP (5 mM), MPA (1 mM), or BSA (10 mg/ml) for various periods of time (0–120 s). Aliquots (500 μl) of the solutions were sampled at various time points and subjected to acid hydrolysis, and the levels of the modified amino acids were determined by HPLC as described below.

Spectral Characterization of NO_2^-/HOCl Reaction Intermediates—To characterize the intermediate(s) produced by reaction of NO_2^- and HOCl we have utilized a continuous flow reaction with photodiode array (PDA) spectrophotometric detection. NO_2^- and HOCl (both at 25 mM in 50 mM KH_2PO_4, pH 6.0) were independently pumped into a mixing junction at a flow rate of 0.3 ml/min. Upon mixing, the reaction effluent was immediately directed into the flow cell of a Waters 996 PDA detector, and the absorbance spectrum of the reaction products was continuously monitored over the range 300–400 nm. In some cases the reaction medium was supplemented with 25% methanol (HPLC grade) in order to compare the absorbance spectra with that of authentic Cl-NO_2 in methanol, as described above.

HPLC Analysis of Reaction Products—All reaction mixtures were analyzed by HPLC, using a 5-μm Spherisorb ODS-2 reverse-phase C-18 column. Samples containing HPA were analyzed directly following experiments without sample preparation by isocratic elution from the column with a mobile phase consisting of 100 mM KH_2PO_4 (pH 3.5)/methanol (70/30, v/v) and UV detection at 274 nm. Samples including NAT, NAP, or BSA were first hydrolyzed in 6 M HCl at 110 °C in sealed glass vials for 4 and 24 h, respectively, to obtain the free amino acids and their modified products. The hydrolysates were then dried using a Centrivap (Labconco) and resuspended in the appropriate mobile phase. Tyrosine, NO_2-Tyr, and Cl-Tyr were analyzed by isocratic elution from the column with 50 mM KH_2PO_4 (pH 3.0)/methanol (92/8, v/v) and subsequent UV detection at 274 nm (15). Dityrosine was detected simultaneously by on-line fluorescence detection using a Waters 470 scanning fluorescence detector (excitation, 284 nm; emission, 410 nm) (15). Phenylalanine (Phe) and *ortho*-, *meta*-, and *para*-chlorophenylalanine (*o*-, *m*-, and *p*-Cl-Phe) were separated on the same column cited above, utilizing a mobile phase consisting of 50 mM KH_2PO_4 (pH 3.0)/methanol (85/15, v/v) with UV detection at 220 nm. Peaks were identified and quantitated using authentic external standards. Peak identity was determined by adding to the sample the authentic compound to establish a match in the HPLC retention time. Peak identity was confirmed using a Waters 996 PDA detector. A spectral match between the authentic chemical and the sample analyte of greater than 90% constituted positive identification.

RESULTS

Interactions of $^{\bullet}NO$, NO_2^-, and HOCl—Aliquots of HPA solutions purged with $^{\bullet}NO$ were reacted with HOCl at various time points to determine if a species is formed under these conditions that is capable of nitrating this model phenolic compound. NO_2-HPA was immediately formed upon HOCl addition, and the yield increased in a manner dependent on the length of time the solution had been purged with $^{\bullet}NO$. The extent of NO_2-HPA formation was significantly lower under deoxygenated (N_2-sparged) conditions (Fig. 1*A*), suggesting that autoxidation of $^{\bullet}NO$ is involved in the reaction with HOCl. $^{\bullet}NO$ rapidly autoxidizes to NO_2^- when bubbled through air-saturated solutions, whereas formation of NO_2^- is significantly diminished under deoxygenated conditions (Fig. 1*B*). As shown in Fig. 1, *A* and *B*, the formation of NO_2-HPA parallels NO_2^- production, suggesting that a nitrating species is formed by reaction of HOCl with NO_2^- or an intermediate species formed during $^{\bullet}NO$ autoxidation.

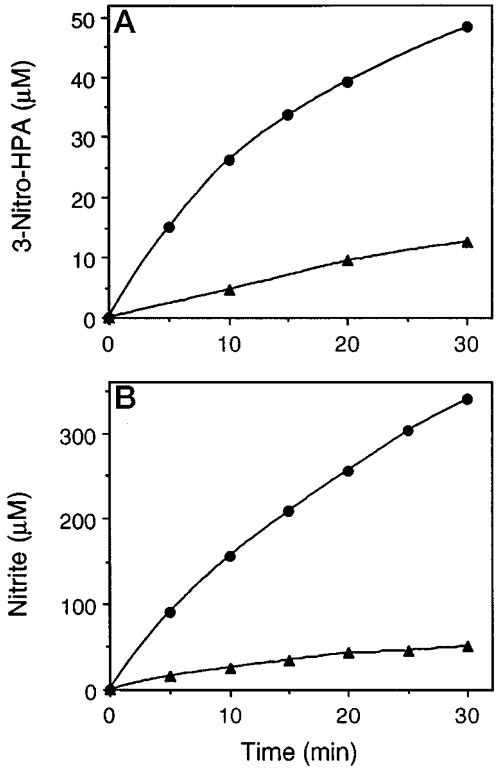

FIG. 1. **Oxygen dependence of HPA nitration by the interaction of $^{\bullet}NO$ and HOCl.** Solutions of HPA (5 mM in 100 mM KH_2PO_4, pH 7.4) were continuously bubbled with $^{\bullet}NO$ (20 ml/min) and reacted with HOCl (1 mM) at various time points under oxygenated (●) and deoxygenated (▲) conditions. The formation of NO_2-HPA (*A*) and NO_2^- (*B*) were determined spectrophotometrically as described under "Experimental Procedures." The data points represent the means of duplicate determinations and are representative of at least two separate experiments.

Modification of HPA and Tyrosine by NO_2^-/HOCl Reaction—A series of experiments were designed using HPA as a substrate to determine whether NO_2^- reacts with HOCl to produce a nitrating species. Indeed, the addition of HOCl to solutions containing NO_2^- and HPA resulted in the immediate (<1 s) formation of a persistent yellow color indicative of phenolic nitration. The presence of NO_2-HPA was confirmed by subsequent HPLC analysis and detection by PDA. Nitration of HPA by the reaction of NO_2^- and HOCl in solution was found to be pH-dependent (Fig. 2). Maximal formation of NO_2-HPA from this reaction occurred at neutral pH, whereas its formation decreased at increasingly acidic or basic pH values and is independent of ionic strength (10–200 mM phosphate) (data not shown). The pH profile of NO_2-HPA formation by the reaction of NO_2^- and HOCl (Fig. 2) indicates that the reaction involves HOCl and *not* ClO^-, because of the rapid decrease in nitration at pH > 7.5 (the pK_a of HOCl). Since the pK_a of NO_2^- is approximately 3.4, it is NO_2^-, and *not* HNO_2, that is the reacting species at all of the pH levels we have studied (pH 5.0–8.5). The decreasing yield of NO_2-HPA at low pH is similar to that determined for the reaction of tyrosine with $^{\bullet}NO_2$ (15, 44) or $ONOO^-$ (15, 16) and may well be due to the lower reactivity of the phenol relative to the phenolate species.

Reaction of NO_2^- (1 mM) and HOCl (1 mM) converted approximately 4% of HPA to NO_2-HPA, similar to the reported yields of NO_2-HPA obtained from reaction of $ONOO^-$ (1 mM) with this substrate (16). The reaction of NO_2^- alone with HPA did not yield NO_2-HPA at any of the pH values studied herein. In the absence of NO_2^-, HOCl directly converted HPA into Cl-HPA at

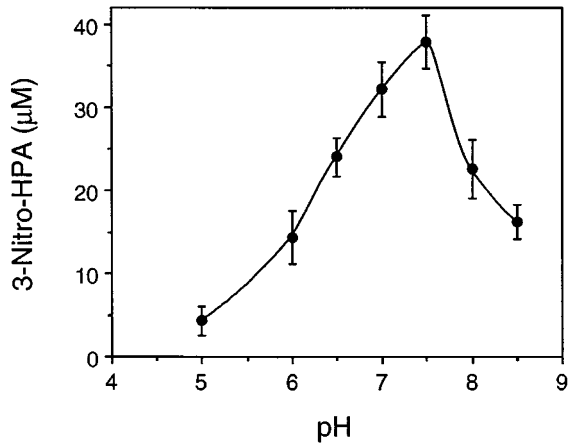

FIG. 2. **The effect of pH on the yield of NO_2-HPA by reaction of HPA with NO_2^- and HOCl.** Mixtures of HPA (1 mM) and NO_2^- (1 mM) in 100 mM KH_2PO_4 were adjusted to the appropriate pH and reacted with a bolus of HOCl (final concentration of 1 mM) at 25 °C. The reaction was allowed to proceed for 10 min and was then quenched with excess GSH (2 mM). The quantitative yield of NO_2-HPA was determined directly by HPLC. The data points are expressed as the mean ± S.D. of four separate experiments.

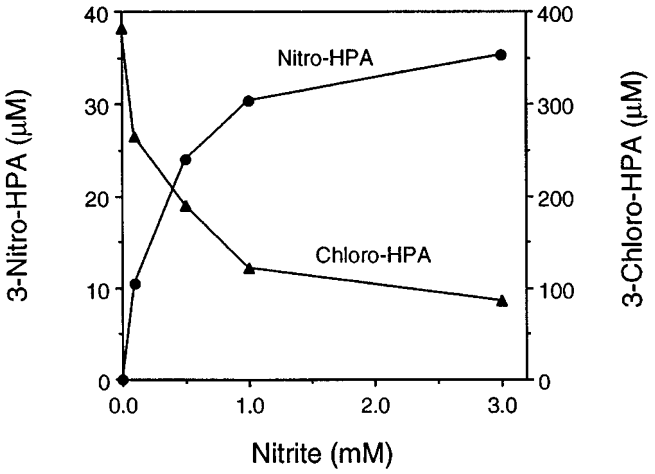

FIG. 3. **The effect of NO_2^- concentration on the yield of NO_2-HPA and Cl-HPA from the reaction of NO_2^- and HOCl with HPA.** HPA solutions (1 mM in 100 mM KH_2PO_4, pH 7.4) containing NO_2^- at various concentrations were exposed to HOCl (1 mM) as a bolus addition. The reactions were allowed to proceed for 10 min, at which time excess GSH was added to scavenge residual HOCl. The yields of NO_2-HPA (●) and Cl-HPA (▲) from the reaction were determined directly by HPLC as described under "Experimental Procedures." Data points are expressed as means of three separate experiments.

nearly 40% yield. Increasing the concentration of NO_2^- (0.1–3.0 mM) in the reaction mixture decreased the yield of Cl-HPA and resulted in a concomitant increase in the formation of NO_2-HPA (Fig. 3), indicating that NO_2^- competed with HPA for reaction with HOCl. When initial NO_2^- and HOCl concentrations were equal (1 mM), the extent of chlorination was approximately 4-fold higher than nitration. Addition of NO_2^- in excess over HOCl (up to 3-fold) did not significantly increase the yield of NO_2-HPA, suggesting that the nitrating species is produced by a 1:1 reaction of HOCl with NO_2^-. Similarly, excess NO_2^- did not significantly decrease formation of Cl-HPA, suggesting that the product formed by this reaction is an efficient chlorinating agent as well as a nitrating species.

We also used NAT as a substrate for the reaction of NO_2^- with HOCl to simulate tyrosine residues in proteins. As shown in Table I, qualitatively and quantitatively similar results were obtained. However, whereas HOCl alone caused very small amounts of dityrosine to be produced (<0.8 μM), the combined addition of NO_2^- and HOCl induced a 15-fold increase in dityrosine formation. Since dityrosine is formed by the combination of two tyrosyl radicals, this result indicated that the reaction between NO_2^- and HOCl produces a species that is capable of carrying out a one-electron oxidation of tyrosine to form the tyrosyl radical. Reactions of NAT with $ONOO^-$ and NO_2BF_4 were also studied in order to compare the nitration mechanisms with that of NO_2^-/HOCl. Although the reaction of $ONOO^-$ (1 mM) with NAT led to NO_2-Tyr levels that were 2-fold higher than that achieved by NO_2^-/HOCl, the levels of dityrosine were nearly identical. The NO_2^+ species (NO_2BF_4) reacted with NAT to form NO_2-Tyr but in lower yields than with either $ONOO^-$ or NO_2^-/HOCl treatments. The reaction of NO_2BF_4 with NAT also yielded relatively high levels of dityrosine, suggestive of tyrosyl radical intermediates in its mechanism of tyrosine nitration.

Modification of Tyrosine in BSA by NO_2^-/HOCl—Reaction of HOCl with solutions of BSA containing NO_2^- resulted in NO_2-Tyr formation, but more slowly than with pure HPA or NAT as substrate. However, when NO_2^- and HOCl were allowed to react just before the addition to BSA (using a dual syringe pump), rapid formation of NO_2-Tyr was observed in a dose-dependent manner (Fig. 4*A*). A small amount of dityrosine (approximately 1 μM) could also be detected in BSA treated in this manner. The species produced by the reaction of NO_2^- and

TABLE I
Comparative modification of NAT by reactions of HOCl, HOCl/NO_2^-, $ONOO^-$ and NO_2^+

N-Acetyltyrosine (5 mM) was dissolved in phosphate buffer (pH 7.4, 100 mM) and reacted with the various reagents (all at 1 mM) as described under "Experimental Procedures." The yields of NO_2-Tyr, Cl-Tyr, and dityrosine were determined following acid hydrolysis of the *N*-acetylated derivatives. Values are the mean ± S.D. of four separate experiments.

Reaction	NO_2-Tyr	Cl-Tyr	Dityrosine
	μM	μM	μM
N-Acetyltyrosine			
$+NO_2^-$	ND[a]	ND	ND
+HOCl	ND	652 ± 42	0.8 ± 0.2
$+HOCl/NO_2^-$	40 ± 4	312 ± 24	14.3 ± 2.3
$+ONOO^-$	86 ± 7	ND	13.2 ± 3.1
$+NO_2BF_4$	9 ± 2	ND	5.9 ± 0.7

[a] ND, not detected.

HOCl also reacted with BSA to produce relatively high levels of Cl-Tyr (Fig. 4*B*). However, when NO_2^- was omitted from one of the syringes (replaced by phosphate buffer), significantly higher levels of Cl-Tyr were detected in BSA, again suggesting that NO_2^- was reacting with HOCl. No detectable levels of these modified tyrosine products were found in acid hydrolysates of control (nonexposed) solutions of BSA.

Characterization of NO_2^-/HOCl Reaction Product(s)—To determine the identity of the product(s) formed by reaction of NO_2^- with HOCl we utilized a continuous flow dual pump PDA system. The spectrum of NO_2^- at pH 6.0 (50 mM KH_2PO_4) under continuous flow through the PDA detector shows a single absorbance maximum at approximately 370 nm (not shown). The addition of HOCl to the continuous flow apparatus via a separate pump leads to the degradation of the NO_2^- absorption and the concomitant formation of a series of maxima observed between 320 and 420 nm (Fig. 5*A*). Johnson and Margerum (45) have studied the reaction of NO_2^- with HOCl and have suggested that nitryl chloride ($Cl\text{-}NO_2$) is a product. Authentic $Cl\text{-}NO_2$ was synthesized as described under "Experimental Procedures," and the absorbance spectrum of this species (in methanol, a polar solvent for which $Cl\text{-}NO_2$ is more stable as compared with aqueous conditions) is shown in Fig. 5*C* for comparison and shows a series of absorption maxima between

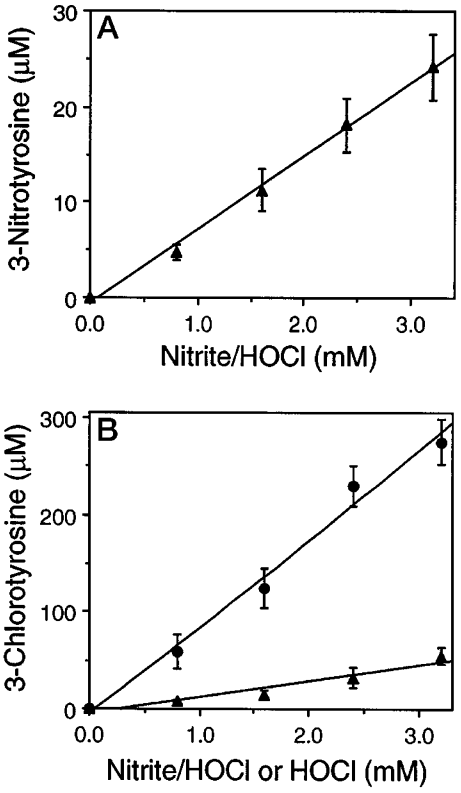

FIG. 4. **Nitration and chlorination of tyrosine residues in BSA exposed to the products of the reaction between NO_2^- and HOCl.** Equal concentrations of HOCl and NO_2^- (or HOCl alone) were mixed together using an automated dual syringe pump, as described under "Experimental Procedures," and allowed to react as a small droplet with a continuously stirred solution of BSA (10 mg/ml). The volume of each droplet added to the solution was calibrated, and the final concentration of the reactants in solution was calculated (0–3.2 mM). The reactions were allowed to proceed for 15 min, at which time excess GSH was added to quench the reaction. The yields of NO_2-Tyr (*A*) and Cl-Tyr (*B*) in BSA by NO_2^-/HOCl combined exposure (▲) or by HOCl alone (●) were determined using HPLC following acid hydrolysis. All data points are expressed as the mean ± S.D. of at least three separate experiments.

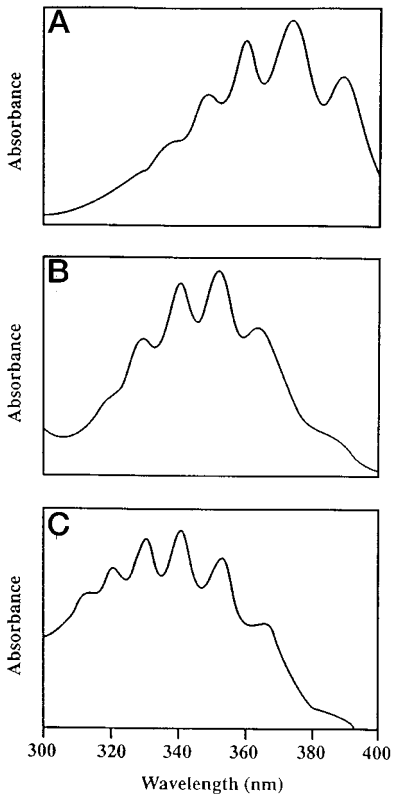

FIG. 5. **Continuous flow UV-visible PDA detection and characterization of the product(s) formed by reaction of NO_2^- with HOCl.** Solutions of NO_2^- and HOCl (both 25 mM in 50 mM KH_2PO_4, pH 6.0) were independently pumped into a mixing junction and allowed to flow directly into a PDA detector immediately after mixing. A typical absorbance spectrum (300–400 nm) of the product(s) formed under these conditions is shown in *A*. Supplementation of the NO_2^- and HOCl solutions with 25% methanol led to a 20-nm hypsochromic shift in the absorbance spectrum (*B*). The absorbance spectrum of synthetic nitryl chloride (Cl-NO_2) in methanol is shown *C*.

320 and 400 nm, characteristic of that for Cl-NO_2 reported previously (43). The addition of methanol to the reaction mixture of NO_2^- and HOCl caused a hypsochromic shift (20 nm) in the absorption spectrum (Fig. 5*B*) without affecting the characteristic series of maxima observed in the absence of methanol. Although the absorbance spectra of authentic Cl-NO_2 and that determined for the product of the reaction between NO_2^- and HOCl show much similarity, the slight differences may be due to the different conditions for which the spectra were obtained (100% methanol for Cl-NO_2, and 25% methanol for NO_2^-/HOCl reaction) and to interference of unreacted NO_2^- in the spectrum of the NO_2^-/HOCl reaction product. Hence, we can conclude that the product formed by this reaction shows characteristics similar to those of Cl-NO_2.

Modification of NAT and Tyrosine Residues in BSA by Cl-NO_2—Since Cl-NO_2 is proposed to be formed by the reaction of NO_2^- with HOCl (45) and the absorption spectrum of the product formed by this reaction suggested the potential formation of Cl-NO_2 in our studies, NAT and BSA were exposed to synthetic Cl-NO_2 in order to compare its reactivity to the species produced by the NO_2^-/HOCl reaction. Exposure of a solution of NAT (5 mM) to a stream of gaseous Cl-NO_2 led to the formation of Cl-Tyr, NO_2-Tyr, and dityrosine to an extent dependent on the duration of exposure (Fig. 6). Formation of Cl-Tyr and dityrosine reached maximum levels at 40 and 50 s respectively, after which the products were decomposed upon further exposure to Cl-NO_2. The loss of Cl-Tyr is likely due to the formation of dichloro-Tyr, as has been shown for the chlorination of tyrosine by Cl_2 gas (46). In contrast, the formation of NO_2-Tyr continued to increase over the entire exposure period, suggesting that NO_2-Tyr is stable under Cl-NO_2 reaction conditions.

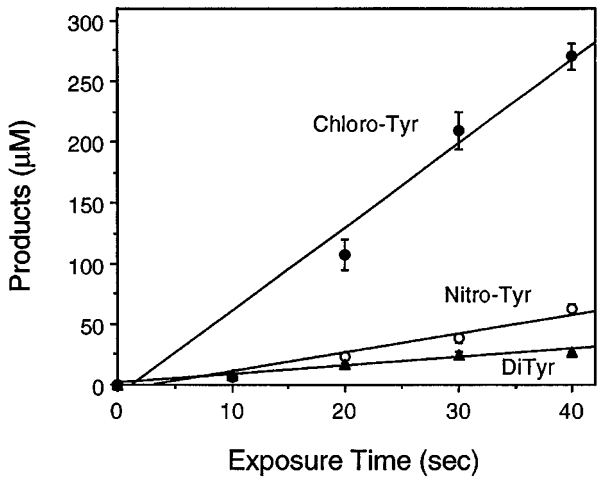

FIG. 6. **Modification of NAT by synthetic Cl-NO_2.** Solutions of NAT (5 mM, in 100 mM KH_2PO_4, pH 7.4) were exposed to gaseous Cl-NO_2 as described under "Experimental Procedures." The yields of NO_2-Tyr (○), Cl-Tyr (●), and dityrosine (▲) were determined by HPLC following acid hydrolysis to liberate the free amino acid and its modified products. All data points are representative of duplicate determinations and are expressed as means ± S.D. of three separate experiments.

TABLE II
Modification of tyrosine residues in BSA exposed to synthetic Cl-NO_2

Solutions of BSA (10 mg/ml) were exposed to Cl-NO_2 as described under "Experimental Procedures." At various time points, aliquots (250 μl) of the BSA solution were taken and subjected to acid hydrolysis to liberate free amino acids and their modified products. The products were identified and quantitated using HPLC. Values represent the mean ± S.D. of three separate experiments.

Exposure Time	NO_2-Tyr	Cl-Tyr	Dityrosine
s	μM	μM	μM
0	ND	ND	ND
30	3 ± 0.2	35 ± 4	0.4 ± 0.1
60	118 ± 14	101 ± 8	2.9 ± 0.3
90	508 ± 35	44 ± 9	2.6 ± 0.2
120	883 ± 28	10 ± 1	1.3 ± 0.1

[a] ND, not detected.

The ratio of Cl-Tyr to NO_2-Tyr formed by Cl-NO_2 averaged 5:1 during the early segment of exposure (20–40 s), and is similar to the ratio of 4:1 obtained by reaction of NO_2^-/HOCl with NAT.

To determine whether the reactions studied with NAT as substrate are relevant to reactions with intact proteins, gaseous Cl-NO_2 was bubbled through solutions of BSA (10 mg/ml). The time-dependent formation of NO_2-Tyr, Cl-Tyr, and dityrosine in BSA exposed to Cl-NO_2 is summarized in Table II. The profile of modified tyrosines was qualitatively similar to that observed for the reaction of NAT with Cl-NO_2, except that the yields of the products were lower, especially at the early time points. Whereas the rapid consumption of NAT by Cl-NO_2 was initiated immediately, the initial loss of tyrosine residues in BSA exposed to Cl-NO_2 was less dramatic, potentially because of competitive reactions with other targets in BSA. The slower initial rate of tyrosine loss in BSA paralleled the oxidation of free sulfhydryl groups in BSA. However, a much more rapid loss of tyrosine ensued following complete depletion of free sulfhydryl groups in BSA (data not shown). The data suggest that reaction of Cl-NO_2 with other amino acid residues (*i.e.*, cysteine, methionine, and lysine) and/or the nonspecific oxidation of the peptide backbone are also important.

Mechanistic Characterization of Nitration and Chlorination Reactions—Since dityrosine is a significant product of the reaction between tyrosine and NO_2^-/HOCl, it is likely that radical species are involved in the reaction mechanisms. To test this hypothesis, we utilized the *O*-methylated derivative of HPA, MPA, a substrate that cannot form phenolic radicals. Fig. 7 compares the absorbance spectra of the products formed when NO_2^-/HOCl or NO_2^+ was reacted with MPA at pH 7.4. NO_2^+, derived from the nitryl salt NO_2BF_4, appears capable of nitrating MPA (pH 7.4) by electrophilic aromatic substitution giving rise to a strong absorbance maximum at 380 nm (Fig. 7, *spectrum C*). However, the reaction of NO_2^-/HOCl with MPA failed to give rise to an absorbance in this region (Fig. 7, *spectrum B*), suggesting that formation of a phenolic radical is an obligatory step in the nitration mechanism involved in this reaction pathway. Reaction of synthetic Cl-NO_2 or HOCl with MPA produced a product(s) with a spectrum nearly identical to that obtained for the NO_2^-/HOCl reaction (data not shown) and suggested that Cl-NO_2 was also incapable of nitrating MPA, implicating radical intermediates in the nitration mechanism of this species. The increased absorbance between 290 and 340 nm observed in both of these cases could be due to chlorination of MPA, suggesting that intermediate phenoxyl radical formation is not a compulsory step in aromatic chlorination.

NAP was used as a model substrate to examine in more detail the mechanisms of aromatic chlorination by the product(s) formed by reaction of NO_2^- with HOCl. Exposure of NAP to HOCl, NO_2^-/HOCl, or Cl-NO_2 led to the formation of *o*-Cl-Phe, *m*-Cl-Phe, and *p*-Cl-Phe to differing extents as illustrated in Fig. 8. Treatment of NAP with HOCl alone led to the formation of *o*-Cl-Phe, *m*-Cl-Phe, and *p*-Cl-Phe in a ratio of 1.00/0.20/0.85. Reactions of NAP with NO_2^-/HOCl or synthetic Cl-NO_2 led to the formation of the *o*-Cl-Phe, *m*-Cl-Phe, and *p*-Cl-Phe isomers in a ratio of 1.0/0.35/0.83 or 1.0/0.40/0.83, respectively. Whereas the *o*-Cl-Phe:*p*-Cl-Phe ratios for all three treatments are nearly identical, an increase in the proportion of *m*-Cl-Phe formed by NO_2^-/HOCl and Cl-NO_2 compared with HOCl alone was observed. The ratios of *o*-Cl-Phe to *m*-Cl-Phe for HOCl, NO_2^-/HOCl, and Cl-NO_2 are 5.0, 2.8, and 2.5, respectively. The high proportion of *o*-Cl-Phe and *p*-Cl-Phe isomers by all of the reactions is indicative of electrophilic aromatic substitution reactions and is consistent with previous studies utilizing a variety of chlorinating agents (47). The nearly 2-fold increase in the *m*-Cl-Phe isomer by reactions of NAP with both NO_2^-/HOCl and Cl-NO_2 suggest a less selective mechanism of chlorination more typical of radical reactions. It is noteworthy that the reaction of NAP with NO_2^-/HOCl or Cl-NO_2 did not form detectable levels of nitrated products.

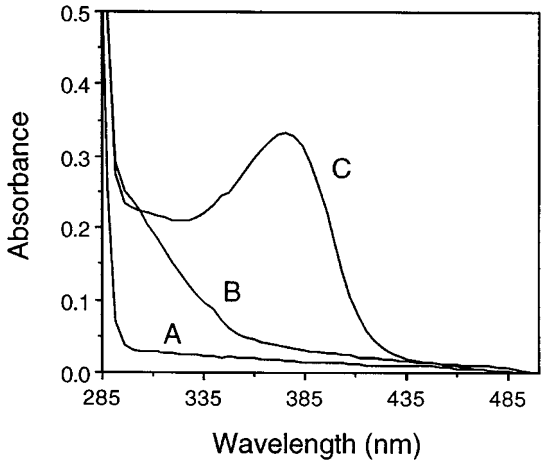

FIG. 7. **Absorbance spectra of the products formed by reaction of MPA with various nitrating species.** Solutions of MPA (*A*) (1 mM in 100 mM KH_2PO_4, pH 7.4) were reacted with NO_2^-/HOCl (*B*) or NO_2^+ (NO_2BF_4) (*C*) by bolus addition of the reactive species at a final concentration of 1 mM. The reactions were allowed to proceed for 20 min, and the absorbance spectrum was measured in a quartz cuvette at pH 7.4. The absorbance maximum at approximately 380 nm in *spectrum C* may be indicative of MPA nitration by NO_2^+. The reaction of authentic nitryl chloride (Cl-NO_2) or HOCl alone with MPA yielded absorbance spectra nearly identical to *spectrum B* (NO_2^-/HOCl treatment) (not shown).

DISCUSSION

Although the mechanisms of biomolecular damage and pathology induced by individual inflammatory oxidants are in general well characterized, an understanding of the complex interactions of ROS and RNS that are likely to occur at sites of inflammation is only just beginning to emerge. The studies reported herein show that the interactions of RNS and HOCl may be important under inflammatory conditions *in vivo*. We have shown that NO_2^-, the autoxidation product of $^{\bullet}NO$ in biological fluids, reacts with HOCl to produce a species that can nitrate, chlorinate, and dimerize biologically relevant phenolic compounds such as tyrosine, both free and within protein. The detection of NO_2-Tyr in a variety of pathologic states (21–23) has been used to indicate the formation of $ONOO^-$ *in vivo*. However, reaction of tyrosine with the products of the NO_2^-/HOCl reaction also forms NO_2-Tyr. Hence, our results suggest that NO_2-Tyr should not be regarded as a specific marker of $ONOO^-$ formation, but only as a marker of RNS.

Mechanism of NO_2^-/HOCl Reaction—It has long been thought (48) that the reaction of NO_2^- with HOCl represented a

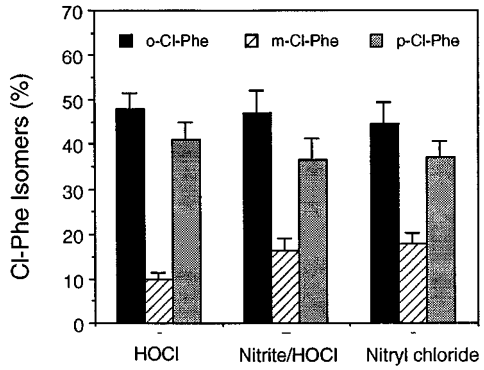

FIG. 8. **The effect of different chlorinating species on the relative distribution of positional isomers of ring-chlorinated phenylalanine.** Solutions of NAP (1 mM in 100 mM KH_2PO_4, pH 7.4) were exposed to HOCl, NO_2^-/HOCl, or synthetic Cl-NO_2 as described under "Experimental Procedures." The yields of *o*-Cl-Phe (*solid bars*), *m*-Cl-Phe (*hashed bars*), and *p*-Cl-Phe (*shaded bars*) were determined by HPLC following acid hydrolysis. The values are expressed as percentages of the total chlorination due to all three isomers. The data represent means ± S.D. of three separate experiments.

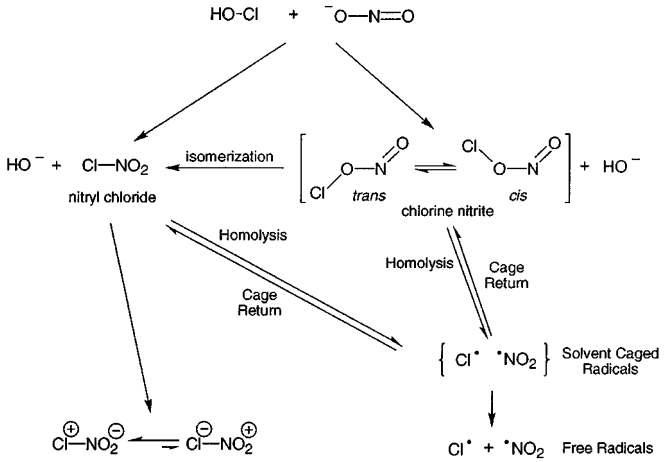

FIG. 9. **Proposed mechanism for the reaction of NO_2^- with HOCl, leading to the formation of reactive intermediates capable of nitrating, chlorinating, and dimerizing aromatic amino acids.**

classical example of an oxygen atom transfer reaction producing NO_3^-.

$$NO_2^- + HOCl \rightarrow HCl + NO_3^-$$

REACTION 2

However, this type of mechanism does not easily explain the nitration and chlorination reactions observed in our studies. Our data suggest a more complex mechanism involving the formation of reactive nitrating and chlorinating intermediates. One-electron oxidation of NO_2^- by HOCl, producing the reactive radical species $Cl^\bullet$ and $^\bullet NO_2$ is one possible pathway. Since HOCl is a poor one-electron oxidant, having an estimated one-electron reduction potential (E'_0) in the range of +0.17 to +0.26 V at pH 7 (38), it is unlikely that a one-electron oxidation mechanism contributes, since the E value for the $^\bullet NO_2/NO_2^-$ couple is approximately +1.04 V (49). In contrast, HOCl is a strong two-electron oxidant (E'_0 = +1.08 V) (38) and would favor the conversion of NO_2^- to the nitryl cation (NO_2^+) or an "NO_2^+-like" species. In addition to a direct two-electron oxidation of NO_2^- by HOCl, a bimolecular substitution reaction between these two reactants could be involved. In fact, contrary to the reaction mechanism previously reported (48), Johnson and Margerum (45) have suggested that HOCl reacts with NO_2^- by Cl^+ transfer, rather than O atom transfer, to yield the intermediate Cl-NO_2, which then hydrolyzes to NO_3^-.

The absorbance spectrum of the product of the reaction between NO_2^- and HOCl (Fig. 5*B*) was found to be similar to that of authentic Cl-NO_2 (Fig. 5*C*). The spectrum of the product(s) of the NO_2^-/HOCl reaction is typical of alkyl nitrites (R-ONO) (50) and therefore could also indicate the formation of a Cl–O-bonded species. In fact, the transfer of Cl^+ to the negatively charged oxygen atom in NO_2^- is likely and would produce the transient intermediate species Cl-ONO. It is possible that both reactions occur (Fig. 9), the extent to which each pathway initially predominates under neutral aqueous conditions is not known. Cl-ONO can exist as both the *cis*- and *trans*-rotamers (Fig. 9), where *ab initio* calculations predict that the energy difference between the two rotamers is approximately 3 kcal/mol, with the *cis* rotamer being the more stable (51). An analogy can be drawn between Cl-ONO and HO-ONO (peroxynitrous acid), where the energy difference between *cis*- and *trans*-HO-ONO is also calculated to be approximately 3 kcal/mol (52). Once formed, Cl-ONO can readily isomerize to Cl-NO_2 (53). We propose that intermediate Cl-ONO can isomerize in aqueous solution to Cl-NO_2 by at least two mechanisms (Fig. 9): 1) intramolecular rearrangement of *trans*-Cl-ONO involving migration of the chlorine atom to the nitrogen atom forming Cl-NO_2, or 2) unimolecular homolysis of the Cl–O bond in Cl-ONO to form a geminate pair of solvent-caged radicals $Cl^\bullet$ and $^\bullet NO_2$, which undergo cage return to either reform Cl-ONO or by recombination to form Cl-NO_2 (Fig. 9). Some of the solvent-caged $Cl^\bullet$ and $^\bullet NO_2$ can escape as "free" radicals and could potentially explain, in part, the radical mechanisms involved in the nitration reactions we observed in the NO_2^-/HOCl reaction. Since Cl-NO_2 is predicted to be 10.7 and 13.8 kcal/mol lower in energy than *cis*- and *trans*-Cl-ONO (51), respectively, the isomerization of Cl-ONO to Cl-NO_2 is a favorable process that shifts the equilibrium toward Cl-NO_2. Isomerization of *cis* Cl-ONO to Cl-NO_2 is probably not likely, because the large size of the chlorine atom, which would presumably preclude the migration of the chlorine atom to the nitrogen atom and, hence, the *trans*-rotamer of Cl-ONO, is probably the species that isomerizes to Cl-NO_2, analogous to the decomposition of *trans*-peroxynitrous acid (*trans*-HO-ONO). Whereas the isomerization of *trans*-HO-ONO leads to nitric acid (HO-NO_2), an unreactive end product, isomerization of Cl-ONO produces another highly reactive species (Cl-NO_2). Hence, Cl-ONO and the product of isomerization, Cl-NO_2, may both be reactive oxidants with nitrating and chlorinating activity.

Decomposition Products of Cl-NO_2 as Reactive Intermediates—We have shown that the product(s) of the reaction between NO_2^- and HOCl, authentic Cl-NO_2, or the NO_2^+ species (NO_2BF_4) react with tyrosine to form NO_2-Tyr and dityrosine. Although none of these reactants are themselves radicals, formation of dityrosine suggests the involvement of intermediate tyrosyl radicals. The nitration of aromatic compounds by NO_2^+ is often thought to be a classical electrophilic aromatic substitution reaction, but there is strong evidence implicating electron transfer reactions and radical intermediates in these pathways (54). This reaction mechanism involves electron transfer from the aromatic to NO_2^+, followed by radical pair collapse, and it would explain the detection of dityrosine in our studies. Hence, we are unable to distinguish between a nitration mechanism involving $^\bullet NO_2$ or NO_2^+ based solely on the formation of dityrosine. However, a divergence in the characteristics of the reaction mechanisms between NO_2^+ and the reactive nitrating species formed by the reaction of NO_2^- with HOCl is evident in their reactions with MPA, the *O*-methylated derivative of HPA,

a substrate incapable of forming phenoxyl radicals. Whereas NO_2^+ appears capable of nitrating MPA, both the product(s) of the NO_2^-/HOCl reaction and synthetic Cl-NO_2 fail to do so. Similarly, the inability of NO_2^-/HOCl and Cl-NO_2 to nitrate phenylalanine further argues against NO_2^+ as the species involved in tyrosine nitration.

There is evidence suggesting that the reaction of Cl-NO_2 with alkenes and aromatic compounds involves homolytic processes yielding free radical intermediates (42), probably involving both Cl$^\bullet$ and $^\bullet NO_2$. Collis *et al.* (43) have found that Cl-NO_2 decomposes at room temperature by homolysis to form Cl_2 and $^\bullet NO_2$ as shown in Reaction 3, whereby these spontaneous decomposition products may be responsible, at least in part, for the chlorinating and nitrating behavior of Cl-NO_2 in our experiments. We suggest that phenolic nitration mediated by the NO_2^-/HOCl reaction involves $^\bullet NO_2$.

$$2\ \text{Cl-NO}_2 \rightarrow (2\ \text{Cl}^\bullet \rightarrow \text{Cl}_2) + (2\ ^\bullet\text{NO}_2 \rightleftharpoons \text{N}_2\text{O}_4)$$

REACTION 3

While the nitration reactions we observed appear to be radical-mediated, chlorination of aromatic amino acids such as phenylalanine (Fig. 8) appears to be executed largely by electrophilic aromatic substitution. In general, chlorination of aromatic compounds by HOCl, *tert*-butyl hypochlorite, and Cl_2 has been shown to be mediated by an ionic rather than a free radical mechanism (47). The nearly 2-fold increase in the relative formation of the *m*-Cl-Phe isomer by reactions of phenylalanine with both NO_2^-/HOCl and Cl-NO_2 (Fig. 8), however, suggests the potential contribution of a less selective mechanism of chlorination, potentially involving Cl$^\bullet$. An active chlorinating species common to HOCl and Cl-NO_2 appears to be Cl_2. In fact, the formation of Cl_2 from HOCl and Cl-NO_2 can be rationalized and would explain the similarities in their chlorinating ability. HOCl is in equilibrium with Cl_2 in aqueous solution as shown in Reaction 4. The formation of Cl_2 from Cl-NO_2 has been proposed to occur by 1) the homolysis of two molecules of Cl-NO_2 to form two Cl$^\bullet$ which combine to form Cl_2 (Reaction 3), and 2) the reaction of Cl-NO_2 with H_2O (43) as shown in Reaction 5.

$$\text{HOCl} + \text{H}^+ + \text{Cl}^- \rightleftharpoons \text{Cl}_2 + \text{H}_2\text{O}$$

REACTION 4

$$3\ \text{Cl-NO}_2 + \text{H}_2\text{O} \rightarrow \text{Cl}_2 + \text{NOCl} + 2\ \text{NO}_3^- + 2\ \text{H}^+$$

REACTION 5

Although convincing evidence suggests an electrophilic substitution mechanism for these chlorination reactions, the possibility of a mechanism involving the addition of Cl$^\bullet$ to the aromatic ring cannot be excluded for reactions involving Cl-NO_2 or NO_2^-/HOCl.

Direct Reactions of Cl-NO_2/Cl-ONO with Tyrosine—The mechanisms of chlorination and nitration discussed thus far have primarily involved species derived from the decomposition of either Cl-NO_2 or Cl-ONO. However, as predicted by the stoichiometry of Reactions 3 and 5, these pathways are particularly favored when Cl-NO_2 or Cl-ONO are present at high concentrations. *In vivo*, however, Cl-NO_2 and Cl-ONO would be expected to be produced at rates that may favor the direct reaction of either species with biological substrates that are present in relative excess. In nonpolar organic solvents Cl-NO_2 has been shown to be an efficient agent for the nitration of aromatic compounds of intermediate reactivity (55). However, an increase either in the reactivity of the aromatic substrate (from benzene to phenol) or in the polarity of the solvent causes a marked decrease in the nitrating efficiency of Cl-NO_2 and a concomitant increase in the yield of chlorinated products (56). In fact, Obermeyer *et al.* (57) argued against the localization of a positive charge on the "nitryl" group of Cl-NO_2, where the structural characteristics of Cl-NO_2 contrast those of typical stable nitryl salts (*i.e.*, $NO_2^+BF_4^-$). Hence, reactions involving activated aromatic substrates such as tyrosine coupled with aqueous conditions would increase aromatic chlorination by Cl-NO_2, suggesting a change from $Cl^-NO_2^+$ character to a species with considerable $Cl^+NO_2^-$ character. Our data suggest that Cl-NO_2 has significant Cl^+ character in aqueous solution, and it is this functionality of Cl-NO_2 that dictates its reactivity.

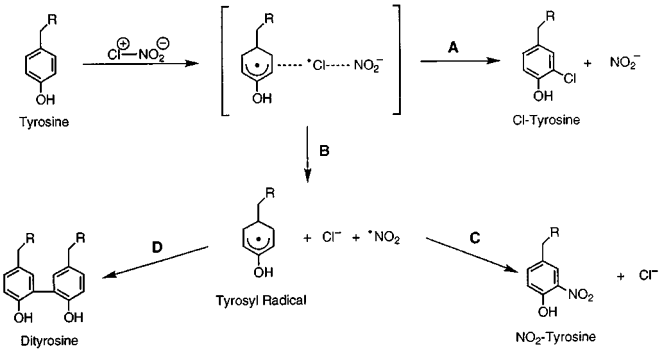

FIG. 10. **Proposed mechanisms for the direct reactions of tyrosine with Cl-NO_2.** The direct reaction of Cl-NO_2 with tyrosine proceeds by electron transfer from tyrosine to $Cl^+NO_2^-$, resulting in an intermediate radical pair (tyrosyl radical–Cl$^\bullet$). Radical pair collapse leads to the formation of Cl-Tyr and NO_2^- (*A*) as major products. Dissociation of the complex from the solvent cage allows Cl$^\bullet$ to oxidize NO_2^- to $^\bullet NO_2$ (*B*), which can combine with simultaneously formed "free" tyrosyl radical to yield NO_2-Tyr (*C*). Dityrosine formation can be envisaged by the combination of two tyrosyl radicals (*D*).

We propose that $Cl^+NO_2^-$ can react directly with tyrosine via electron transfer to yield an intermediate radical pair (tyrosyl radical–Cl$^\bullet$–NO_2^-) (Fig. 10). Radical pair collapse of this complex leads to the rapid formation of Cl-Tyr and NO_2^- (Fig. 10, *reaction A*), and is the major product formed by this reaction. This electron transfer-mediated reaction mechanism is analogous to the nitration of phenolic substrates by NO_2^+ (54). Dissociation of the radical pair complex and subsequent oxidation of NO_2^- by Cl$^\bullet$ (a strongly oxidizing species, E'_0 of Cl$^\bullet$/Cl^- = +2.2–2.6 V (Ref. 49)) results in the formation of "free" tyrosyl radical and $^\bullet NO_2$ (Fig. 10, *reaction B*). Tyrosyl radical and $^\bullet NO_2$ can rapidly combine to yield NO_2-Tyr ($k = 3 \times 10^9\ \text{M}^{-1}\ \text{s}^{-1}$ (Ref. 44)), and dityrosine formation can be envisaged by the combination of two tyrosyl radicals (Fig. 10, *reactions C* and *D*). This proposed mechanism predicts that the yields of the various tyrosine modification products will be on the order of Cl-Tyr $\gg$ NO_2-Tyr $>$ dityrosine, consistent with the data presented herein. The proposed reaction pathway also illustrates the dependence of radical intermediates in the nitration of phenolic compounds by Cl-NO_2, as suggested by our data.

Since Cl-ONO is a potential transient intermediate in the formation of the reactive species Cl-NO_2 (Fig. 9), part of the reactivity of NO_2^-/HOCl may be attributed to Cl-ONO. Analogous to a proposed mechanism of ONOOH reactivity (9, 58), a vibrationally excited intermediate derived from *trans*-Cl-ONO (Cl-ONO*) may be formed during its isomerization to Cl-NO_2 and contribute to nitration and chlorination of tyrosine by direct reaction. The reaction mechanisms we propose for Cl-NO_2 and Cl-ONO are analogous to those recently determined for ONOOH, whereby both direct and indirect reactions with oxidizable substrates can occur (59). A more detailed examina-

tion of the reaction kinetics and thermodynamic considerations is necessary in order to elucidate which of the proposed mechanisms predominate.

Physiological Relevance and Biological Implications—The activation and accumulation of neutrophils at sites of tissue injury, leading to the formation of HOCl and other ROS/RNS, is an essential feature of inflammation. Our data suggest that the reaction of HOCl with NO_2^-, derived from •NO produced by other phagocytes (30), endothelial cells (2), or epithelial cells (37), may be a contributing pathway operative in tissue injury at sites of inflammation. Moreover, since Cl-NO_2 is conceivably formed *in vivo* and is capable of nitrating tyrosine residues, our findings may imply a role for this reaction pathway where NO_2-Tyr is detected in cases of acute inflammatory lung injury (21), atherosclerosis (22), and rheumatoid arthritis (23), a phenomena previously ascribed to $ONOO^-$ formation. In fact, increased levels of NO_2^- have been observed in similar cases (36, 60, 61) and further suggest the potential involvement of this pathway *in vivo*. Recent studies have demonstrated that myeloperoxidase, the phagocytic enzyme that catalyzes HOCl formation, is a component of sputum from cystic fibrosis patients (62), as well as other inflammatory lung diseases, and of human atherosclerotic tissue (63), underscoring the potential importance of HOCl in the pathology of each of these cases. We propose, then, that tyrosine nitration by Cl-ONO and/or Cl-NO_2, formed by the reaction of NO_2^- with HOCl, represents an important and additional mechanism for inflammation-mediated tyrosine nitration *in vivo*, independent of $ONOO^-$ formation.

Our findings indicate that NO_2^- may not be an appropriate marker of •NO production by neutrophils or at sites of inflammation, since it is potentially removed by reaction with simultaneously produced HOCl. Determination of •NO production in tissues and fluids of patients with acute and chronic inflammation, as measured by NO_2^- "accumulation," is likely a gross underestimate. Moreover, NO_2^- has been shown to modulate the bactericidal activity of HOCl, its mechanism proposedly mediated by direct reaction of these two species (39, 41). Our findings suggest, then, that the reaction product, Cl-NO_2, is a strongly oxidizing species that may serve as an antimicrobial agent in its own right.

An analogous reaction between hypobromous acid, a product formed by oxidation of Br^- catalyzed by eosinophil peroxidase (64), and NO_2^- forming Br-ONO and/or Br-NO_2 can be envisioned. In fact, Reaction 6 may represent a general mechanism by which hypohalous acids (HOX) react with NO_2^- to produce species capable of oxidizing biological molecules.

$$HOX + NO_2^- \rightarrow X\text{-}ONO + X\text{-}NO_2 + OH^-$$

REACTION 6

Collectively, these reaction pathways could therefore represent an important host defense mechanism and a novel pathway for inflammation-mediated tissue injury.

Conclusions—We report here that NO_2^- and HOCl react to form the reactive intermediates Cl-NO_2 and/or Cl-ONO, species that are capable of nitrating, chlorinating, and dimerizing phenolic compounds such as tyrosine. Our data suggest that NO_2-Tyr is not necessarily a specific marker of $ONOO^-$ formation *in vivo* and that Cl-NO_2 and Cl-ONO may be important and previously unconsidered oxidants produced at sites of inflammation.

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Note added in proof—Upon further investigation of NO_2^+-mediated nitration of MPA using HPLC, we were unable to detect nitration by the nitryl salt NO_2BF_4 under neutral aqueous conditions. Hence, we cannot rule out the contribution of an NO_2^+ species to nitration events observed with NO_2^-/HOC1 or C1-NO_2 in our experiments reported herein.

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