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 (NO2−), the autoxidation product of nitric oxide (NO), with hypochlorous acid (HOCI) forms reactive intermediate species that are also capable of nitrating phenolic substrates such as tyrosine and 4-hydroxy-phenylacetic acid, with maximum yields obtained at physiological pH. Monitoring the reaction of NO2− with HOCI by continuous flow photodiode array spectrophotometry indicates the formation of a transient species with spectral characteristics similar to those of nitryl chloride; Cl-NO2, chlorine nitrite; ROS, reactive oxygen species; RNS, reactive nitrogen species; HPLC, high pressure liquid chromatography; PDA, photodiode array.

Nitrogen monoxide (nitric oxide, 'NO') is produced by a variety of cells through the activity of constitutive and inducible forms of nitric oxide synthase (1). ‘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 'NO are normally quite low (nanomolar), local 'NO concentrations have been shown to increase to levels ranging from 4 to 30 μM under pathologic conditions (4, 5).

‘NO reacts at a near diffusion-controlled rate with superoxide (O2−) (k = 6.7 × 109 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 ‘NO (7, 8). At physiological pH the protonated form of ONOO−, peroxynitrous acid (ONOOH) (pKa = 6.8), is highly unstable and readily decomposes to nitrate (NO2−). 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 (NO2) and the hydroxyl radical (‘OH), or 3) by heterolytic dissociation to form the nitryl cation (nitronium ion, NO2+) (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 (NO2−-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 biological...
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$_2^-$) and hydrogen peroxide (H$_2$O$_2$) as a result of the activation of the respiratory burst oxidase (26). In the case of neutrophils, some of the H$_2$O$_2$ 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.

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

**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 NO through the activation of inducible nitric oxide synthase; however, the ability of human neutrophils to produce NO is debated (31). Once formed, NO can react with several biological targets, primarily thought to involve heme-iron, hyperreactive sulfhydryls and protein radicals (32–34). 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 NO$_2$$^-$ and dinitrogen trioxide (N$_2$O$_3$) (35). In fact, NO$_2^-$ has been used as a marker of 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 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 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 (CI-HPA), 4-methoxyphenylacetic acid (MPA), and nitronium tetrafluoroborate (NO$_2$BF$_4$; 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, 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$_2$O$_2$. 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.

NO$_2$ Experiments—NO$_2$ (100 ppm in O$_2$-free N$_2$) was bubbled through continuously stirred 100-ml solutions of phosphate buffer (100 mM KH$_2$PO$_4$, 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 NO$_2$ 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 NO, and NO$_2$ product was determined spectrophotometrically using Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine, and 2.5% H$_3$PO$_4$) (41). In separate experiments, HPA (5 mM) was added to the buffer solutions prior to NO$_2$ exposure. At various time points, 0.5-ml aliquots of the solution were withdrawn from the reaction mixture and immediately reacted in the HOCl (1 mM). After 10 min, the reaction was adjusted to pH 10–11 with 1 M NaOH. NO$_2$-HPA was measured spectrophotometrically at 430 nm (ε = 4400 M$^{-1}$ cm$^{-1}$).

Nitration and Chlorination Reactions—Buffered solutions (100 mM KH$_2$PO$_4$ 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$_2$PO$_4$ prior to experimentation. Nitric acid was dosed to give a desired concentration (0.1–3.0 µM) 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$^-$ (1.2 µM NaNO$_2$ or NO$_2$BF$_4$ (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 µM) 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-m1 solutions of BSA (10 mg/ml) in 100 mM KH$_2$PO$_4$ (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$_2$PO$_4$ (pH 7.4) so as to allow exposure of BSA to HOCl alone under the same conditions. Following addition of the BSA 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 CI-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 CI-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, CI-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 CI-NO$_2$ is essentially the same previously described (42, 43) with slight modification. Sulfuric acid (61% solution) was added dropwise to vigorously stirred nitric acid (50 g) at 0°C in a 500-m1 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 iceacetonite. 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 CI-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 CI-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 oxygen 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-NO2 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-NO2 showed a series of characteristic absorption maxima between 300 and 400 nm, similar to that reported previously (43).

Nitryl Chloride Exposures—CI-NO2 (10 ml) was placed in a 50-ml sparging flask immersed in a cooling dry ice/aceton bath. A stream of N2 gas was allowed to flow through a glass tube fitted with a fritted glass fitting, which was submerged into the undiluted CI-NO2, and was bubbled through the liquid at a flow rate of 75 ml/min. Gaseous CI-NO2 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. CI-NO2 in N2 gas was allowed to bubble through the 100 ml 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). All 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 NO2-/HOCl Reaction Intermediates—To characterize the intermediate(s) produced by reaction of NO2 and HOCl we have utilized a continuous flow reaction with photodiode array (PDA) spectrophotometric detection. NO2 and HOCl (both at 25 mM in 50 mM KH2PO4, 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-NO2 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 KH2PO4 (pH 3.0)/methanol (70/30, v/v) and UV detection at 274 nm. Samples including NAT, NAP, or BSA were first hydrolyzed in 6 N 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, NO2-Tyr, and CI-Tyr were analyzed by isocratic elution from the column with 50 mM KH2PO4 (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) and UV detection at 274 nm (12). The hydrolysates of tyrosine, ortho-m-chlorophenylalanine (p-c-Cl-Phe) and para-chlorophenylalanine (p-Cl-Phe) were separated on the same column cited above, utilizing a mobile phase consisting of 50 mM KH2PO4 (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 confirmed 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 NO, NO2, and HOCl—All aliquots of HPA solutions purged with 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. NO2-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 NO. The extent of NO2-HPA formation was significantly lower under deoxygenated (N2-sparged) conditions (Fig. 1A), suggesting that autoxidation of NO is involved in the reaction with HOCl. NO rapidly autoxidizes to NO2 when bubbled through air-saturated solutions, whereas formation of NO2 is significantly diminished under deoxygenated conditions (Fig. 1B). As shown in Fig. 1, A and B, the formation of NO2-HPA parallels NO2 production, suggesting that a nitrating species is formed by reaction of HOCl with NO2 or an intermediate species formed during NO autoxidation. Modification of HPA and Tyrosine by NO2/HOCI Reaction—A series of experiments were designed using HPA as a substrate to determine whether NO2 reacts with HOCl to produce a nitrating species. Indeed, the addition of HOCl to solutions containing NO2 and HPA resulted in the immediate (<1 s) formation of a persistent yellow color indicative of phenolic nitrilation. The presence of NO2-HPA was confirmed by subsequent HPLC analysis and detection by PDA. Nitrilation of HPA by the reaction of NO2 and HOCl in solution was found to be pH-dependent (Fig. 2). Maximal formation of NO2-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 NO2-HPA formation by the reaction of NO2 and HOCl (Fig. 2) indicates that the reaction involves HOCl and not ClO−, because of the rapid decrease in nitrilation at pH > 7.5 (the pKa of HOCl). Since the pKa of NO2 is approximately 3.4, it is NO2, and not HNO2, that is the reacting species at all of the pH levels we have studied (pH 5.0–8.5). The decreasing yield of NO2-HPA at low pH is similar to that determined for the reaction of tyrosine with NO2 (15, 44) or ONOO− (15, 16) and may well be due to the lower reactivity of the phenol relative to the phenolate species.

Reactions of NO2 (1 mM) and HOCl (1 mM) converted approximately 4% of HPA to NO2-HPA, similar to the reported yields of NO2-HPA obtained from reaction of ONOO− (1 mM) with this substrate (16). The reaction of NO2 alone with HPA did not yield NO2-HPA at any of the pH values studied herein. In the absence of NO2, HOCl directly converted HPA into CI-HPA at
Nitration and Chlorination of Tyrosine by NO2/HOCl Reaction

![Graph showing pH effect on NO2-HPA yield](image)

Fig. 2. The effect of pH on the yield of NO2-HPA by reaction of HPA with NO2 and HOCl. Mixtures of HPA (1 mM) and NO2 (1 mM) in 100 mM KH2PO4 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 NO2-HPA was determined directly by HPLC. The data points are expressed as the mean ± S.D. of four separate experiments.

![Graph showing NO2 concentration effect on yield](image)

Fig. 3. The effect of NO2 concentration on the yield of NO2-HPA and Cl-HPA from the reaction of NO2 and HOCl with HPA. HPA solutions (1 mM in 100 mM KH2PO4, pH 7.4) containing NO2 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 NO2-HPA (●) and Cl-HPA (▲) from the reaction were determined directly by HPLC as described under "Experimental Procedures." The data points are expressed as means of three separate experiments.

![Table]

| Reaction          | NO2-Tyr | Cl-Tyr | Dityrosine |
|-------------------|---------|--------|------------|
| N-Acetyltyrosine  | ND      | ND     | ND         |
| +NO2              | ND      | ND     | ND         |
| +HOCl             | ND      | 652 ± 42 | 0.8 ± 0.2   |
| +HOCl/NO2         | 40 ± 4  | 312 ± 24 | 14.3 ± 2.3 |
| +ONO2             | 86 ± 7  | ND     | 13.2 ± 3.1 |
| +NO2BF4           | 9 ± 2   | ND     | 5.9 ± 0.7  |

ND, not detected.

We also used NAT as a substrate for the reaction of NO2 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 NO2 and HOCl induced a 10-fold increase in dityrosine formation. Since dityrosine is formed by the combination of two tyrosyl radicals, this result indicated that the reaction between NO2 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 NO2BF4 were also studied in order to compare the nitration mechanisms with that of NO2/HOCl. Although the reaction of ONOO− (1 mM) with NAT led to NO2-Tyr levels that were 2-fold higher than that achieved by NO2/HOCl, the levels of dityrosine were nearly identical. The NO2-Tyr species (NO2BF4) reacted with NAT to form NO2-Tyr but in lower yields than with either ONOO− or NO2/HOCl treatments. The reaction of NO2BF4 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 NO2/HOCl—Reaction of HOCl with solutions of BSA containing NO2 resulted in NO2-Tyr formation, but more slowly than with pure HPA or NAT as substrate. However, when NO2 and HOCl were allowed to react just before the addition to BSA (using a dual syringe pump), rapid formation of NO2-Tyr was observed in a dose-dependent manner (Fig. 4A). 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 NO2 and HOCl also reacted with BSA to produce relatively high levels of CI-Tyr (Fig. 4B). However, when NO2 was omitted from one of the syringes (replaced by phosphate buffer), significantly higher levels of CI-Tyr were detected in BSA, again suggesting that NO2 was reacting with HOCl. No detectable levels of these modified tyrosine products were found in acid hydrolysates of the N-acetylated derivatives. Values are the mean ± S.D. of four separate experiments.

Characterization of NO2/HOCl Reaction Products—To determine the identity of the product(s) formed by reaction of NO2 with HOCl, we utilized a continuous flow dual pump PDA system. The spectrum of NO2 at pH 6.0 (50 mM KH2PO4) under continuous flow through the PDA detector shows a single absorption 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 NO2 absorption and the concomitant formation of a series of maxima observed between 320 and 420 nm (Fig. 5A). Johnson and Margerum (45) have studied the reaction of NO2 with HOCl and have suggested that nitryl chloride (Cl-NO2) is a product. Authentic Cl-NO2 was synthesized as described under "Experimental Procedures," and the absorbance spectrum of this species (in methanol, a polar solvent for which Cl-NO2 is more stable as compared with aqueous conditions) is shown in Fig. 5C for comparison and shows a series of absorption maxima between

Table I

| Reaction          | NO2-Tyr | Cl-Tyr | Dityrosine |
|-------------------|---------|--------|------------|
| N-Acetyltyrosine  | ND      | ND     | ND         |
| +NO2              | ND      | ND     | ND         |
| +HOCl             | ND      | 652 ± 42 | 0.8 ± 0.2   |
| +HOCl/NO2         | 40 ± 4  | 312 ± 24 | 14.3 ± 2.3 |
| +ONO2             | 86 ± 7  | ND     | 13.2 ± 3.1 |
| +NO2BF4           | 9 ± 2   | ND     | 5.9 ± 0.7  |

ND, not detected.
320 and 400 nm, characteristic of that for Cl-NO\textsubscript{2} reported previously (43). The addition of methanol to the reaction mixture of NO\textsubscript{2} and HOCl caused a hypsochromic shift (20 nm) in the absorption spectrum (Fig. 5B) without affecting the characteristic series of maxima observed in the absence of methanol. Although the absorbance spectra of authentic Cl-NO\textsubscript{2} and that determined for the product of the reaction between NO\textsubscript{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\textsubscript{2}, and 25% methanol for NO\textsubscript{2}/HOCl reaction) and to interference of unreacted NO\textsubscript{2} in the spectrum of the NO\textsubscript{2}/HOCl reaction product. Hence, we can conclude that the product formed by this reaction shows characteristics similar to those of Cl-NO\textsubscript{2}.

Modification of NAT and Tyrosine Residues in BSA by Cl-NO\textsubscript{2}—Since Cl-NO\textsubscript{2} is proposed to be formed by the reaction of NO\textsubscript{2} with HOCl (45) and the absorption spectrum of the product formed by this reaction suggested the potential formation of Cl-NO\textsubscript{2} in our studies, NAT and BSA were exposed to synthetic Cl-NO\textsubscript{2} in order to compare its reactivity to the species produced by the NO\textsubscript{2}/HOCl reaction. Exposure of a solution of NAT (5 mM) to a stream of gaseous Cl-NO\textsubscript{2} led to the formation of Cl-Tyr, NO\textsubscript{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\textsubscript{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\textsubscript{2} gas (46). In contrast, the formation of NO\textsubscript{2}-Tyr continued to increase over the entire exposure period, suggesting that NO\textsubscript{2}-Tyr is stable under Cl-NO\textsubscript{2} reaction conditions.
Table II
Modification of tyrosine residues in BSA exposed to synthetic Cl-NO2
Solutions of BSA (10 mg/ml) were exposed to Cl-NO2 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 (s) | NO2-Tyr μM | Cl-Tyr μM | Dityrosine μM |
|-------------------|------------|-----------|---------------|
| 5                 | 4.2 ± 0.1  | 1.7 ± 0.1 | 0.5 ± 0.1     |
| 30                | 1.4 ± 0.1  | 0.6 ± 0.1 | 0.2 ± 0.1     |
| 60                | 0.8 ± 0.1  | 0.2 ± 0.1 | 0.1 ± 0.1     |
| 90                | 0.4 ± 0.1  | 0.1 ± 0.1 | 0.05 ± 0.01   |
| 120               | 0.2 ± 0.1  | 0.05 ± 0.01 | 0.02 ± 0.005 |

ND, not detected.

The ratio of Cl-Tyr to NO2-Tyr formed by Cl-NO2 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 NO2 with HOCl with NAT.

To determine whether the reactions studied with NAT as substrate are relevant to reactions with intact proteins, gaseous Cl-NO2 was bubbled through solutions of BSA (10 mg/ml). The time-dependent formation of NO2-Tyr, Cl-Tyr, and dityrosine in BSA exposed to Cl-NO2 is summarized in Table II. The profile of modified tyrosines was qualitatively similar to that observed for the reaction of NAT with Cl-NO2 except that the yields of the products were lower, especially at the early time points. Whereas the rapid consumption of NAT by Cl-NO2 was initiated immediately, the initial loss of tyrosine residues in BSA exposed to Cl-NO2 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-NO2 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 NO2/HOCI, 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 NO2/HOCI or NO2 was reacted with MPA at pH 7.4. NO2 derived from the nitryl salt NO2BF4 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 NO2/HOCI 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-NO2 or HOCI with MPA produced a product(s) with a spectrum nearly identical to that obtained for the NO2/HOCI reaction (data not shown) and suggested that Cl-NO2 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 NO2 with HOCI. Exposure of NAP to HOCI, NO2/HOCI, or Cl-NO2 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 NO2/HOCI or synthetic Cl-NO2 led to the formation of the o-Cl-Phe, m-Cl-Phe, and p-Cl-Phe isomers in a ratio of 1.00/0.35/0.83 or 1.00/0.40/0.83, respectively. Whereas the o-Cl-Phe-pCl-Phe ratios for all three treatments are nearly identical, an increase in the proportion of m-Cl-Phe formed by NO2/HOCI and Cl-NO2 compared with HOCl alone was observed. The ratios of o-Cl-Phe to m-Cl-Phe for HOCl, NO2/HOCI, and Cl-NO2 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 NO2/HOCI and Cl-NO2 suggests a less selective mechanism of chlorination more typical of radical reactions. It is noteworthy that the reaction of NAP with NO2/HOCI or Cl-NO2 did not form detectable levels of nitrated products.

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 HOCI may be important under inflammatory conditions in vivo. We have shown that NO2, the autoxidation product of NO in biological fluids, reacts with HOCI 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 NO2-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 NO2/HOCI reaction also forms NO2-Tyr. Hence, our results suggest that NO2-Tyr should not be regarded as a specific marker of ONOO− formation, but only as a marker of RNS.

Mechanism of NO2/HOCI Reaction—It has long been thought (48) that the reaction of NO2 with HOCl represented a...
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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 KH₂PO₄, pH 7.4) were exposed to HOCl, NO₂/HOCl, or synthetic Cl-NO₂ 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. 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 KH₂PO₄, pH 7.4) were exposed to HOCl, NO₂/HOCl, or synthetic Cl-NO₂ 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.

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 NO₂ by HOCI, producing the reactive radical species Cl⁻ and NO₂⁻ is one possible pathway. Since HOCI is a poor one-electron oxidant, having an estimated one-electron reduction potential (E₀₂) 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 NO₂⁻/NO₂ couple is approximately +1.04 V (49). In contrast, HOCI is a strong two-electron oxidant (E₀₂ = +1.08 V) (38) and would favor the conversion of NO₂⁻ to the nitryl cation (NO₂²⁻) or an "NO₂²⁻-like" species. In addition to a direct two-electron oxidation of NO₂ by HOCI, a bimolecular substitution reaction between these two reactants could be involved. In fact, contrary to the reaction mechanism previously reported (48), Johnson and Marгерum (45) have suggested that HOCI reacts with NO₂⁻ by Cl⁻ transfer, rather than O atom transfer, to yield the intermediate Cl-ONO₂⁻, which then hydrolyzes to NO₃⁻.

The absorbance spectrum of the product of the reaction between NO₂⁻ and HOCI (Fig. 5B) was found to be similar to that of authentic Cl-NO₂⁻ (Fig. 5C). The spectrum of the product(s) of the NO₂⁻/HOCI reaction is typical of alkyl nitrites (R-ONO₂⁻) (50) and therefore could also indicate the formation of a Cl⁻-bonded species. In fact, the transfer of Cl⁻ to the negatively charged oxygen atom in NO₂⁻ is likely and would produce the transient intermediate species Cl-ONO₂⁻. 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-ONO₂⁻ 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-ONO₂⁻ and HO-ONO₂⁻ (peroxynitrous acid), where the energy difference between cis- and trans-HO-ONO₂⁻ is also calculated to be approximately 3 kcal/mol (52). Once formed, Cl-ONO₂⁻ can readily isomerize to Cl-NO₂⁻ (53). We propose that intermediate Cl-ONO₂⁻ can isomerize in aqueous solution to Cl-NO₂⁻ by at least two mechanisms (Fig. 9): 1) intramolecular rearrangement of trans-Cl-ONO₂⁻ involving migration of the chlorine atom to the nitrogen atom forming Cl-NO₂⁻, or 2) unimolecular homolysis of the Cl-O bond in Cl-ONO₂⁻ to form a geminate pair of solvent-caged radicals Cl⁻ and NO₂⁻, which undergo cage return to either reform Cl-ONO₂⁻ or by recombination to form Cl-NO₂⁻ (Fig. 9). Some of the solvent-caged Cl⁻ and NO₂⁻ can escape as "free" radicals and could potentially explain, in part, the radical mechanisms involved in the nitration reactions we observed in the NO₂⁻/HOCI reaction. Since Cl-NO₂⁻ is predicted to be 10.7 and 13.8 kcal/mol lower in energy than cis- and trans-Cl-ONO₂⁻ (51), respectively, the isomerization of Cl-ONO₂⁻ to Cl-NO₂⁻ is a favorable process that shifts the equilibrium toward Cl-NO₂⁻. Isomerization of cis Cl-ONO₂⁻ to Cl-NO₂⁻ 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-ONO₂⁻, is probably the species that isomerizes to Cl-NO₂⁻, analogous to the decomposition of trans-peroxynitrous acid (trans-HO-ONO). Whereas the isomerization of trans-HO-ONO leads to nitric acid (HO-NO₂⁻), an unreactive end product, isomerization of Cl-ONO₂⁻ produces another highly reactive species (Cl-NO₂⁻). Hence, Cl-ONO₂⁻ and the product of isomerization, Cl-NO₂⁻, may both be reactive oxidants with nitrating and chlorinating activity.

Decomposition Products of Cl-NO₂⁻ as Reactive Intermediates—We have shown that the product(s) of the reaction between NO₂⁻ and HOCI, authentic Cl-NO₂⁻, or the NO₂⁻ species (NO₂BF₄⁻) react with tyrosine to form NO₂⁻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 NO₂⁻ 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 NO₂⁻, followed by radical pair collapse, and it would explain the detection of dityrosine in our studies. Hence, we are unable to distinguish between a nitrination mechanism involving NO₂⁻ or NO₂⁻ based solely on the formation of dityrosine. However, a divergence in the characteristics of the reaction mechanisms between NO₂⁻ and the reactive nitrating species formed by the reaction of NO₂⁻ with HOCI 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$^-$ and NO$_2$. Collis et al. (43) have found that Cl-NO$_2$ decomposes at room temperature by homolysis to form Cl$_2$ and 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 NO$_2$.

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

**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$^-$. 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$^-$ which combine to form Cl$_2$ (Reaction 3), and 2) the reaction of Cl-NO$_2$ with H$_2$O (43) as shown in Reaction 5.

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

**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$^-$ 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$^-$/NO$_2$ character in aqueous solution, and it is this functionality of Cl-NO$_2$ that dictates its reactivity.

We propose that Cl$^-$/NO$_2$ can react directly with tyrosine via electron transfer to yield an intermediate radical pair (tyrosyl radical–Cl$^-$) (Fig. 10). 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$^-$ to oxidize NO$_2$ to 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).

![Proposed mechanisms for the direct reactions of tyrosine with Cl-NO$_2$.](Image)

**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$^-$). 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$^-$ to oxidize NO$_2$ to 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).
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Note added in proof—Upon further investigation of NO$_2$-mediated nitrination of MPA using HPLC, we were unable to detect nitrination by the nitryl salt NO$_2$Br$_2$ under neutral aqueous conditions. Hence, we cannot rule out the contribution of an NO$_2$ species to nitrination events observed with NO$_2$/HOCl or Cl-NO$_2$ in our experiments reported herein.

REFERENCES

1. Knowles, R. G., and Moncada, S. (1994) Biochem. J. 298, 249–258
2. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
3. Schmidt, H. H., and Walter, U. (1994) Cell 78, 919–925
4. Hooper, D. C., Ohnishi, S. T., Kean, R., Numagami, Y., Dietzschold, B., and Kropowski, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5312–5316
5. Malinski, T., Zhang, Z. G., and Chopp, M. (1993) Cerebral Blood Flow Metab. 13, 355–358
6. Hsu, R. E., and Padmanab, S. (1993) Free Rad. Res. Commun. 18, 195–199
7. Brunelli, L., Crow, J. P., and Beckman, J. S. (1995) Arch. Biochem. Biophys. 316, 327–334
8. Lipton, S. A., Choi, Y.-B., Pan Z.-H., Lei, S. Z., Chen, H.-S. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993) Nature 364, 626–632
9. Pryor, W. A., and Smedrilo, G. L. (1995) Am. J. Physiol. 268, L699–L722
10. Ischiropoulos, H., and Al-Medrano, A. B. (1995) FEBS Lett. 364, 279–282
11. Pryor, W. A., In, X., and Smedrilo, G. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11173–11177
12. Rad, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Biol. Chem. 266, 4244–4250
13. Halfpenny, E., and Robinson, P. L. (1952) J. Chem. Soc. (Lond.) 939–946
14. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624
15. Van der Vliet, A., Eisner, P. O., O'Neil, C. A., Halliwell, B., and Cross, C. E. (1995) Arch. Biochem. Biophys. 319, 341–349
16. Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) Arch. Biochem. Biophys. 298, 438–445
17. Van der Vliet, A., O'Neil, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994) FEBS Lett. 339, 89–95
18. Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992) Arch. Biochem. Biophys. 298, 446–451
19. Carreras, M. C., Pargament, G. A., Catz, S. D., Poderoso, J. J., and Boveris, A. (1994) FEBS Lett. 341, 65–68
20. Kody, N. W., and Royall, J. A. (1994) Arch. Biochem. Biophys. 310, 352–359
21. Haddad, I. Y., Pataki, G., Hu, P., Galliani, C., Beckman, J. S., and Matalon, S. (1994) J. Clin. Invest. 93, 2417–2423
22. Kooy, J. S., Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994) Biochem. Hoppe-Seyler's 375, 81–88
23. Kaur, H., and Halliwell, B. (1994) FEBS Lett. 350, 9–12
24. Squitrito, G. L., and Pryor, W. A. (1995) Chem. Biol. Interact. 96, 203–206
25. Miller, R. A., and Britigan, B. E. (1995) J. Invest. Med. 43, 39–49
26. Winterbourn, C. C. (1990) in Oxygen Radicals: Systemic Events and Disease Processes (Das, D. K., and Essman, W. B., eds) pp. 31–70, Karger, Basel, Switzerland
27. Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995) J. Biol. Chem. 270, 6154–6158
28. Weiss, S. J., Lampert, M. B., and Test, S. T. (1983) Biochem. J. 218, 21891–21897
29. Salters, L. M., and Kettle, A. J. (1995) J. Biol. Chem. 270, 8706–8711
30. Miles, A. M., Owens, M. W., Milligan, S. J., Johnson, G. G., Fields, J. Z., Ing, T. S., Kottapalli, V., Keshavarzian, A., and Grisham, M. B. (1995) Leukocyte Biol. 56, 616–622
31. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) Science 258, 1898–1902
32. Lepore, M., Fiaran, J.-M., Bobé, P., Lemaire, G., and Henry, Y. (1994) Biochem. Mol. Biol. Int. 34, 23891–23897
33. Eisner, P. O., Butler, J., van der Vliet, A., Cross, C. E., and Halliwell, B. (1995) Biochem. J. 310, 745–749
34. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., and Byrn, R. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8120–8127
35. Farrell, A. J., Blake, D. R., Palmer, R. M. J., and Moncada, S. (1992) Ann. Rheum. Dis. 51, 1219–1222
36. Gaston, B., Reilly, J., Drazen, M. J., Fackler, J., Rammel, P., Arnele, D., Mullins, M. E., Sugabaker, D. J., Chee, C., Singel, D. J., Loscalzo, J., and Stamler, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10975–10981
37. Koppang, O. W. (1994) FEBS Lett. 347, 5–8
38. Kono, Y. (1995) Biochem. Mol. Biol. Int. 36, 275–283
39. Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) Methods Enzymol. 233, 229–240
40. Klebanoff, S. J. (1993) Free Radical Biol. & Med. 14, 351–360
41. Schecter, H., Conrad, F., Daughton, A. L., and Kaplan, R. B. (1952) J. Am. Chem. Soc. 74, 3052–3053
42. Collins, M. J., Gintz, F. P., Goddard, D. R., Hebdon, E. A., and Minkoff, G. J. (1958) J. Chem. Soc. (Lond.) 445–451
43. Prötz, W. A., Mönig, H., Butler, J., and Land, E. J. (1985) Arch. Biochem. Biophys. 243, 125–134
44. Johnson, D. W., and Marginer, D. W. (1991) Inorg. Chem. 30, 4845–4851
45. Nickelsen, M. G., Nweke, A., Scully, F. E., and Ringhand, H. P. (1991) Chem. Res. Toxicol. 4, 94–101
46. Watson, D. W. (1974) J. Org. Chem. 39, 1160–1164
47. Arban, M., and Taube, H. (1958) J. Am. Chem. Soc. 80, 1073–1077
48. Wardman, P. (1989) Phys. Chem. Ref. Data 18, 1671–1755
49. Haszeldine, R. N. (1953) J. Chem. Soc. (Lond.) 2525–2527
50. Lee, T. J. (1994) J. Phys. Chem. 98, 111–115
51. Tsai, J. H.-M., Harrison, J. G., Martin, J. C., Hamilton, T. P., van der Woerd,
Nitration and Chlorination of Tyrosine by NO₂/HOCl Reaction

M., Jablonsky, M. J., and Beckman, J. S. (1994) J. Am. Chem. Soc. 116, 4115–4116
53. Tevault, D. E., and Smardzewski, R. R. (1977) J. Chem. Phys. 67, 3777–3784
54. Perrin, C. L. (1977) J. Am. Chem. Soc. 99, 5516–5518
55. Price, C. C., and Sears, C. A. (1953) J. Am. Chem. Soc. 75, 3276–3277
56. Gintz, F. P., Goddard, D. R., and Collins, M. J. (1958) J. Chem. Soc. (Lond.) 445–451
57. Obermeyer, A., Bormann, H., and Simon, A. (1995) J. Am. Chem. Soc. 117, 7887–7890
58. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) Chem. Res. Toxicol. 5, 834–842
59. Goldstein, S., and Czapski, G. (1995) Inorg. Chem. 34, 4041–4048
60. Gaston, B., Drazen, J. M., Loscalzo, J., and Stamler, J. S. (1994) Am. J. Respil. Crit. Care Med. 149, 538–531
61. Chester, A. H., O’Neil, G. S., Moncada, S., Tadjkarimi, S., and Yacoub, M. H. (1990) Lancet 336, 897–900
62. Mohammed, J. R., Mohammed, S. S., Pawluk, L. J., Bucci, D. M., Baker, N. R., and Davis, W. B. (1988) J. Lab. Clin. Med. 112, 711–720
63. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) J. Clin. Invest. 94, 437–444
64. Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D., and Regiani, S. (1986) Science 234, 200–203