Eosinophil Peroxidase Nitrates Protein Tyrosyl Residues

IMPLICATIONS FOR OXIDATIVE DAMAGE BY NITRATING INTERMEDIATES IN EOSINOPHILIC INFLAMMATORY DISORDERS*

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Eosinophil peroxidase (EPO) has been implicated in promoting oxidative tissue injury in conditions ranging from asthma and other allergic inflammatory disorders to cancer and parasitic/helminthic infections. Studies thus far on this unique peroxidase have primarily focused on its unusual substrate preference for bromide (Br⁻) and the pseudohalide thiocyanate (SCN⁻) forming potent hypohalous acids as cytotoxic oxidants. However, the ability of EPO to generate reactive nitrogen species has not yet been reported. We now demonstrate that EPO readily uses nitrite (NO₂⁻), a major end-product of nitric oxide (NO) metabolism, as substrate to generate a reactive intermediate that nitrates protein tyrosyl residues in high yield. EPO-catalyzed nitration of tyrosine occurred more readily than bromination at neutral pH, plasma levels of halides, and pathophysiologically relevant concentrations of NO₂⁻. Furthermore, EPO was significantly more effective than MPO at promoting tyrosine nitration in the presence of plasma levels of halides. Whereas recent studies suggest that MPO can also promote protein nitration through indirect oxidation of NO₂⁻ with HOCl, we found no evidence that EPO can indirectly mediate protein nitration by a similar reaction between HOBr and NO₂⁻. EPO-dependent nitration of tyrosine was modulated over a physiologically relevant range of SCN⁻ concentrations and was accompanied by formation of tyrosyl radical addition products (e.g. o-o'dityrosine, pulcherosine, trityrosine). The potential role of specific antioxidants and nucleophilic scavengers on yields of tyrosine nitration and bromination by EPO are examined. Thus, EPO may contribute to nitrosylation formation in inflammatory conditions characterized by recruitment and activation of eosinophils.

Eosinophils play a central role in host defenses against a variety of cancers and both parasitic and helminthic infections (1–3). Increased levels of circulating and tissue eosinophils are also implicated in promoting cellular injury during asthma and other allergic inflammatory disorders (1–6). Eosinophils are thought to mediate many of their cytotoxic and tissue-destroying effects through their exceptional ability to generate oxidizing species (1–4, 7–9). Indeed, the respiratory burst of eosinophils generates several times as much superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) as a corresponding number of neutrophils (9, 10). Despite their known proclivity for producing reactive oxygen species and a wealth of clinical evidence linking eosinophils to host defenses and inflammatory tissue injury, structural identification of specific oxidation products formed by these leukocytes is lacking.

Eosinophil peroxidase (EPO), an abundant heme protein secreted from activated eosinophils, plays a central role in oxidant production by eosinophils (1–4, 8, 11, 12). EPO amplifies the oxidizing potential of H₂O₂ produced during the respiratory burst by using it as a co-substrate to generate cytotoxic oxidants. Studies thus far have focused on the unusual substrate preference of EPO for physiological levels of bromide (Br⁻) (8, 13–17) and the pseudohalide thiocyanate (SCN⁻) (18–20), even in the presence of a vast molar excess of chloride (Cl⁻), as is seen in vivo. Studies with proteins incubated in the presence of radioactive Br⁻ or SCN⁻ salts and either activated eosinophils or the EPO-H₂O₂ system have demonstrated covariant incorporation of radiolabel into target proteins (13, 14). However, structural identification of the oxidation products formed on proteins following exposure to EPO-generated oxidants is essentially unexplored. We recently identified 3-bromotyrosine and 3,5-dibromotyrosine as major products of protein oxidation by the EPO-H₂O₂-Br⁻ system (21). The role of brominating oxidants in promoting tissue injury in eosinophilic inflammatory disorders has not yet been established.

Another potential pathway for oxidative tissue damage by eosinophils may involve formation of nitrating intermediates by EPO. Immunohistochemical studies using antibodies raised against nitrotirosine, a global marker for protein damage by reactive nitrogen species, intensely stain eosinophils present in the inflamed lung tissues of asthmatics (22). Klebanoff (23) demonstrated that the antimicrobialicidal properties of eosinophil peroxidase-H₂O₂ systems are enhanced in the presence of nitrite (NO₂⁻), the autodioxidation product of NO. Moreover, recent studies have shown that myeloperoxidase (MPO), a neutrophil- and monocyte-specific peroxidase, can use NO₂⁻ and H₂O₂ as substrates to generate a reactive intermediate capable of nitrating phenolic compounds and protein tyrosyl residues (25–28). However, MPO and EPO are distinct gene products with divergent physical properties. Although both peroxidases contain heme prosthetic groups, structural studies have established that MPO contains a six-coordinate, high spin chlorin,
while EPO possesses a high spin, six-coordinate putorophorin (24). Moreover, although human MPO is tetrameric and comprised of two heavy and two light chains, EPO is dimeric, having only one heavy and one light chain (7, 24). Whether these structural differences underlie the distinct halide and substrate specificities observed between MPO and EPO remains unknown. Furthermore, although increased levels of NO2- and eosinophils have been reported in a variety of conditions, the ability of EPO to generate nitrating intermediates has not yet been explored.

The biological roles and potential targets of mammalian peroxidases are defined by their unique and often nonoverlapping substrate preferences (29, 30). We therefore sought to determine whether the EPO-H2O2 system of eosinophils could promote protein oxidative damage through formation of reactive nitrogen species. We now show that isolated EPO readily uses NO2- as substrate to generate a reactive intermediate that nitrates protein tyrosyl residues in high yield. We demonstrate that EPO is more efficient than MPO at nitrating tyrosine and that EPO-dependent protein nitration is a preferred activity of the enzyme. Finally, we provide evidence that EPO also generates tyrosyl radical and that tyrosine nitration by EPO may involve a tyrosyl radical intermediate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Organic solvents, H2PO4, NaH2PO4, Na2HPO4, and H2O were obtained from Fisher. Methane sulfonic acid and bromine were purchased from Fluka Chemical Co. (Ronkonkoma, NY). Constant boiling HCl was obtained from Pierce. L-[13C6]Tyrosine and L-[315]H2O2 were purchased from Cambridge Isotopes, Inc. (Andover, MA). All other reagents were purchased from Sigma unless otherwise indicated.

**Isolation and Characterization of EPO and MPO**—Porcine EPO was isolated according to the method of Jorg (31) employing guaiacol oxidation as the assay (32). Purity of EPO preparations was assured before use by demonstrating a RZ of >0.9 (A280/A400). SDS-polyacrylamide gel electrophoresis analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating peroxidases are defined by their unique and often nonoverlapping substrate specificities. MPO activity (33). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described (34). Trace levels of contaminating EPO were then removed by passage over a Mono Q column. Purity of isolated MPO was established by demonstrating a RZ of 0.87 (A280/A400). SDS-polyacrylamide gel electrophoresis analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating MPO activity (35). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described (34). Trace levels of contaminating EPO were then removed by passage over a Mono Q column. Purity of isolated MPO was established by demonstrating a RZ of 0.87 (A280/A400). SDS-polyacrylamide gel electrophoresis analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating MPO activity (35). Enzyme concentrations were determined spectrophotometrically utilizing extinction coefficients of 89,000 and 112,000 M−1 cm−1/heme of MPO (35) and EPO (36, 37), respectively. The concentration of the MPO dimer was calculated as half of the indicated concentration of hemelike chromophore.

**General Procedures**—SDS-polyacrylamide gel electrophoresis analysis was performed as described by Laemmli (38). Protein content was measured by the Markwell-modified Lowry assay with bovine serum albumin (BSA) as the standard (39). H2O2 free of Br− and bromate was prepared from liquid bromide the day of use as described (40). HOBr was quantified spectrophotometrically (ε254 = 315 M−1 cm−1) (41) as its conjugate base, hypobromite (OHBr−), immediately prior to use. The concentration of reagent H2O2 (ε254 = 39.4 M−1 cm−1) (42) was determined spectrophotometrically. Production of H2O2 by the glucose/glucose oxidase system was determined by oxidation of Fe(II) and formation of a Fe(III)-thiocyanate complex (25). Western blot analysis of nitrated proteins was determined as described using immunopurified rabbit polyclonal antibody against nitrotyrosine (Upstate Biotechnol-ogy, Inc., Lake Placid, NY) (43). The specificity of the primary antibody was confirmed by blocking immunoreactivity in incubations with either 10 mM nitrotyrosine or 1 mM of the tripeptide Gly-Gly-Tyr-Ala as described (43). o-0-Dityrosine, pulcherosine, trityrosine, and isodesmosine were prepared and quantified as described previously (44). 3-Bromotyrosine and 3,5-dibromotyrosine standards were synthesized from L-tyrosine and isolated by preparative HPLC as recently described (21). [13C6]Ring-labeled analogs of 3-bromotyrosine and 3,5-dibromotyrosine were similarly prepared using L-[13C6]Tyrosine as starting material. UV-visible studies on authentic and EPO-generated 3-nitrotyrosine were performed on a Bio Spectrophotometer (Perkin-Elmer).
Nitrotyrosine Formation by Eosinophil Peroxidase

Fig. 1. Reverse-phase HPLC separation of L-tyrosine oxidation products generated by eosinophil peroxidase. Top, L-tyrosine (500 μM) was incubated with eosinophil peroxidase (57 nM) and H₂O₂ (500 μM) in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. Products were subsequently analyzed by reverse phase HPLC as described under “Experimental Procedures.” Bottom, L-tyrosine (500 μM) was incubated with eosinophil peroxidase (57 nM), H₂O₂ (500 μM), and NO₂⁻ (1 mM) in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. Products were then analyzed by reverse phase HPLC as described under “Experimental Procedures.”

Nitrotyrosine was also analyzed by GC/MS analysis as its n-propyl per-heptafluorobutyl derivative following reduction to 3-aminotyrosine (50). Negative ion chemical ionization GC/MS studies were performed utilizing a Perkin Elmer (Norwalk, CT) TurboMass spectrometer equipped with chemical ionization probe.

RESULTS

3-Nitrotyrosine and Tyrosyl Radical Addition Products Are Formed during L-Tyrosine Oxidation by the Eosinophil Peroxidase-H₂O₂-NO₂⁻ System—The addition of purified EPO and H₂O₂ to reaction buffer (20 mM sodium phosphate, 100 μM diethylenetriaminepentaacetic acid, pH 7.0) supplemented with L-tyrosine generated several new species with distinct retention times on reverse phase HPLC analysis with diode array detection (Fig. 1, upper panel, peaks a–c). The ultraviolet absorption maximum of each peak was nearly indistinguishable from L-tyrosine (λ_max 275–285 nm). LC/MS analyses were consistent with formation of the tyrosyl radical addition products o,o'-dityrosine and isodityrosine (peak a; m/z 361 (M + H⁺)), pulcherosine (peak b; m/z 540 (M + H⁺)), and trityrosine (peak c; m/z 540 (M + H⁺)) (44). Likewise, HPLC analysis with UV and fluorescence detection demonstrated co-migration with authentic synthetic standards prepared as described under
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“Experimental Procedures.” Following the addition of NO2, the reaction mixture developed an intense yellow color. Reverse phase HPLC analysis demonstrated the formation of an additional major (peak I) and minor (peak d) product (Fig. 1, bottom panel) as well as enhanced production of the tyrosyl radical addition products (peaks a-c). The UV-visible spectra of the new products (peaks I and d) both illustrated similar absorbance maxima in the visible wavelength range (λmax 350–355 nm), consistent with nitration of the aromatic amino acid (51). Use of heat-inactivated EPO or omission of either L-tyrosine or H2O2 from the reaction mixture both resulted in no detectable production of the products. The reaction products formed (peak I and peaks a–d) were stable to treatment with acid (4 N HCl at 100 °C for 24 h), incubation at 37 °C with H2O2, and the addition of a molar excess of either reducing agents or nucleophilic scavengers (e.g. NaCNBH3, methionine, 2-mercaptoethanol, taurine, ammonium acetate).

To characterize the structures of the L-tyrosine oxidation products generated by the EPO-H2O2-NO2 system, a variety of analytical techniques were employed. The pH dependence of the UV-visible absorbance spectrum of HPLC-isolated peak I is illustrated in Fig. 2 (upper panel) and is identical to that observed with authentic 3-nitrotyrosine. To confirm the structure of the major L-tyrosine oxidation product (peak I) as 3-nitrotyrosine, LC/MS analysis was performed. The retention time and positive ion mass spectrum of peak I was identical to that of authentic 3-nitrotyrosine and demonstrated a m/z of 227 (M + H)+ (Fig. 2, lower panel). LC/MS analysis of the minor products generated during oxidation of L-tyrosine by the complete EPO-H2O2-NO2 system was consistent with the prior structural assignments of peaks a–c and suggested that peak d was composed of a nitrat analog of o,o′-dityrosine (e.g. 3-nitro-o,o′-dityrosine, m/z 406 (M + H)+). These results, combined with the chemical stability and fluorescence spectra (data not shown) of the compounds formed, suggest that the major compounds generated during oxidation of free L-tyrosine by EPO and H2O2 are tyrosyl radical addition products. In contrast, in the presence of EPO, H2O2, and NO2, 3-nitrotyrosine is the major product formed.

Characterization of Reaction Requirements for EPO-dependent Formation of 3-Nitrotyrosine—The reaction requirements for EPO-catalyzed nitration of L-tyrosine yielding 3-nitrotyrosine are illustrated in Fig. 3. At pH 7.0 and 100 μM L-tyrosine and NO2, 3-nitrotyrosine synthesis by EPO (following the bolus addition of 100 μM H2O2) was linear over the first 15 min and reached a plateau within 30 min (Fig. 3A). At higher L-tyrosine concentrations, the yield of 3-nitrotyrosine decreased, consistent with the competing bimolecular mechanism of o,o′-dityrosine formation. EPO-catalyzed nitration was optimal between pH 6 and 6.5, where approximately 0.35 mol of product was formed for each mole of oxidant consumed (Fig. 3B). A dose-dependent increase in the synthesis of 3-nitrotyrosine was observed over a physiologically relevant range of NO2 concentrations (Fig. 3C). The H2O2 concentration dependence for 3-nitrotyrosine production by EPO yielded a similarly shaped plot and overall yield (Fig. 3D). At high concentrations of H2O2, however, the total amount of 3-nitrotyrosine formed decreased, consistent with either substrate inhibition or an interaction between H2O2 and an intermediate in 3-nitrotyrosine formation (data not shown).

3-Nitrotyrosine Is a Preferred Product of Eosinophil Peroxidase at Physiologically Relevant Concentrations of L-Tyrosine, Halides, and NO2—Thiocyanate (SCN−) and bromide (Br−) are reported to be preferred substrates for EPO (13–16, 18). To estimate the relative substrate preferences of isolated EPO for NO2 versus other reported substrates for the enzyme, we ex-

**Fig. 3. Reaction characteristics of 3-nitrotyrosine formation by the eosinophil peroxidase.** Reactions (0.5 ml) were performed at 37 °C for 60 min in sodium phosphate buffer (50 mM, pH 7.0) supplemented with L-tyrosine (100 μM), H2O2 (100 μM), NO2 (100 μM) and eosinophil peroxidase (57 nM) (unless otherwise indicated). Reactions were terminated by the addition of phenol (1%), and the extent of 3-nitrotyrosine formation was determined as described under “Experimental Procedures.” Results represent the mean values from two or three independent experiments.

A

![Graph A](image1.png)

B

![Graph B](image2.png)

C

![Graph C](image3.png)

D

![Graph D](image4.png)
amine the initial rate of DTNB formation in reaction mixtures containing EPO, TNB, H$_2$O$_2$, and various concentrations of anionic substrates (SCN$^-$, Br$^-$, NO$_2^-$, Cl$^-$) at neutral pH. The relative rates of TNB oxidation observed by EPO were as follows: SCN$^-$ > Br$^-$ > NO$_2^-$ > Cl$^-$ (Fig. 4). Although EPO does not conform to Michaelis-Menten kinetics (e.g. high concentrations of H$_2$O$_2$ inhibit tyrosine nitration), a measure of the relative ability of EPO to discriminate in favor of a particular substrate in the presence of a mixture of competing substrates can be calculated as an apparent specificity constant ($k_x$) for each substrate (33). Because high levels of H$_2$O$_2$ can inhibit oxidant production, we estimated the kinetic parameters under conditions that minimize inhibition and mimic physiological conditions (e.g. neutral pH and low concentrations of H$_2$O$_2$) (Table I). SCN$^-$ was the preferred substrate for EPO, demonstrating an approximately 2.7-fold higher apparent specificity constant than Br$^-$, the next best substrate examined. EPO preferred Br$^-$ approximately 4-fold more than NO$_2^-$ (Table I). Implicit in this type of kinetic analysis is the assumption that the reactive nitrogen species formed by the EPO-H$_2$O$_2$-NO$_2^-$ system oxidize TNB at a rate and stoichiometry comparable with that of other hypohalous acids formed by the enzyme.

As an alternative means of assessing the potential significance of EPO-dependent tyrosine nitration, we performed product analyses of the major L-tyrosine oxidation products formed by the enzyme system (Table I). When plasma levels of L-tyrosine and halides were incubated with isolated EPO, H$_2$O$_2$, and concentrations of NO$_2^-$ as might be observed in inflammatory tissues or fluids (tyrosine = Br$^-$ = NO$_2^-$ = 100 mM, Cl$^-$ = 100 mM; Refs. 25 and 52–56), 3-nitrotyrosine was formed in high yield (Table II). In fact, 3-nitrotyrosine was the major product formed and was present at 2–3-fold greater levels than either 3-bromotyrosine or o,o’-dityrosine (Table II). Despite that vast molar excess of Cl$^-$, no detectable formation of 3-chlorotyrosine was observed under all of the conditions examined, consistent with the low apparent specificity constant observed (Tables I and II). The highest yields of 3-nitrotyrosine production were observed during incubations in the absence of Br$^-$, consistent with the unusual substrate preference of EPO for this halide.

3-Nitrotyrosine Formation by Eosinophil Peroxidase Occurs in the Presence of Nucleophilic Scavengers and Is Inhibited by Ascorbate and Urate—Reactive oxidants and diffusible radical species generated by peroxidases in vivo will encounter a variety of nucleophilic scavengers and antioxidant compounds. To characterize the nature of the reactive halogenating and nitrating intermediates generated by EPO, we incubated the enzyme with H$_2$O$_2$, Br$^-$, and NO$_2^-$ under a variety of conditions and then quantified 3-bromotyrosine and 3-nitrotyrosine production. Again, when L-tyrosine was incubated with EPO, H$_2$O$_2$ (100 mM), and equivalent amounts of Br$^-$ and NO$_2^-$ (100 mM each), 3-nitrotyrosine was the major oxidation product formed and was produced in 2–3-fold higher yield than 3-bromotyrosine (Fig. 5). The addition of methionine, a potent scavenger

![Fig. 4. Eosinophil peroxidase-dependent oxidation of TNB in the presence of Br$^-$, NO$_2^-$, Cl$^-$, or SCN$^-$. Eosinophil peroxidase (14.2 nM) was mixed with 53 μM TNB and the indicated concentrations of Br$^-$ (●), NO$_2^-$ ( ○), Cl$^-$ (△), or SCN$^-$ (■). Reactions were initiated by the addition of 30 μM H$_2$O$_2$, and DTNB formation was monitored spectrophotometrically at 412 nm as described under “Experimental Procedures.” Rates of DTNB formation were corrected for TNB oxidation in the absence of anionic substrate (0.26 μM/min). Data represent the mean values for three independent experiments.](image)

TABLE I

**Kinetic parameters for substrates of eosinophil peroxidase**

The apparent $V_{max}$ and $K_m$ values for chloride, nitrite, bromide, and thiocyanate were determined by linear regression analysis of double reciprocal plots for the rate of TNB formation versus anion concentration for experiments performed at neutral pH and 30 μM H$_2$O$_2$ as in Fig. 4. The catalytic rate constants ($k_x$) were calculated by dividing $V_{max}$ values by the concentration of EPO. The specificity constants ($k_x/k_m$) were determined by dividing $k_{obs}$ by $K_m$.

| Anion  | $V_{max}$ (μM/min) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{x}/k_m$ | $k_{x}/k_m$ for Cl$^-$ |
|--------|--------------------|------------|----------------------|-------------|------------------------|
| Cl$^-$ | 1.45               | 430        | 1.70                 | 4.0         | 1                      |
| NO$_2^-$ | 1.83              | 2.3        | 2.15                 | 9.3 × 10^2  | 240                    |
| Br$^-$ | 3.09               | 0.9        | 3.63                 | 4.0 × 10^3  | 1020                   |
| SCN$^-$ | 13.1               | 1.4        | 15.4                 | 1.1 × 10^4  | 2780                   |

**TABLE II**

**L-tyrosine oxidation by eosinophil peroxidase in the presence of physiological concentrations of Cl$^-$, Br$^-$, and NO$_2^-$**

L-tyrosine (100 μM) was incubated with eosinophil peroxidase (57 nM), H$_2$O$_2$ (100 μM), and the indicated anionic substrates (Br$^-$ = NO$_2^-$ = 100 μM, Cl$^-$ = 100 mM) in 1 ml of sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. The quantities of o,o’-dityrosine, 3-bromotyrosine, 3-nitrotyrosine, and 3-chlorotyrosine formed were then determined by reverse phase HPLC with UV detection as described under “Experimental Procedures.” Results are the mean ± S.D. of three independent experiments. The limit of detection for these analyses is ~0.1 nmol for each analyte examined.

| Reaction mixture | o,o’-Dityrosine | 3-Bromotyrosine | 3-Nitrotyrosine | 3-Chlorotyrosine |
|------------------|---------------|----------------|----------------|-----------------|
| Tyr/EPO/H$_2$O$_2$/NO$_2$/Br$^-$/Cl$^-$ | 1.92 ± 0.08 | 2.83 ± 0.14 | 6.89 ± 0.26 | ND* |
| Without EPO | ND            | ND             | ND             | ND              |
| Without H$_2$O$_2$ | ND          | ND             | ND             | ND              |
| Without Br$^-$/Cl$^-$ | 1.49 ± 0.07 | ND             | ND             | ND              |
| Without Br$^-$/NO$_2^-$ | 3.12 ± 0.29 | ND             | 9.29 ± 1.36   | ND              |
| Without Cl$^-$ | 1.91 ± 0.15  | 2.46 ± 0.02   | 6.33 ± 0.11   | ND              |
| Without Br$^-$ | 2.75 ± 0.43  | ND             | 11.00 ± 0.12  | ND              |

* ND, not detected.
of reactive halogenating species, completely ablated 3-bromotyrosine production. In contrast, the addition of the thiol ether-containing amino acid to the reaction mixture had no significant effect on 3-nitrotyrosine production (Fig. 5). Likewise, the addition of reduced thiol compounds such as glutathione totally inhibited tyrosine bromination but only partially blocked aromatic nitration by the peroxidase. The addition of either ascorbic acid or uric acid potently inhibited both 3-bromotyrosine and 3-nitrotyrosine production. The addition of primary amine-containing species (e.g., N-acetyl lysine) to the reaction mixture did not attenuate either 3-bromotyrosine or 3-nitrotyrosine production. These results are consistent with our recent report that N-monobromamines serve as brominating intermediates for tyrosine (21). They also suggest that the nitrating intermediate formed by the EPO-H$_2$O$_2$-NO$_2$$_{3}^{-}$ system is not scavenged by amine groups. Finally, these results suggest that aromatic nitration reactions may be mediated by EPO in inflammatory fluids and tissues where NO$_2$ levels are increased and antioxidants such as ascorbate and urate may be depleted.

Eosinophil Peroxidase Nitrates Protein Tyrosyl Residues in High Yield—The ability of EPO to nitrate protein tyrosyl residues was first examined by incubating a target protein (e.g., BSA) with the complete EPO-H$_2$O$_2$-NO$_2$$_{3}^{-}$ system and then performing SDS-polyacrylamide gel electrophoresis analysis with Western blot analysis using affinity-purified polyclonal antibody against 3-nitrotyrosine (43). A NO$_2$-dependent increase in the intensity of staining was observed following exposure of the protein to the enzymatic system (Fig. 6). To confirm that 3-nitrotyrosine was produced in proteins exposed to the EPO-H$_2$O$_2$-NO$_2$$_{3}^{-}$ system, protein hydrolysates were directly analyzed for 3-nitrotyrosine production by GC/MS in the negative-ion chemical ionization mode employing synthetic 3-nitro-[13C$_6$]tyrosine as an internal standard, as described under "Experimental Procedures." Ions with the appropriate mass-to-charge ratio (m/z 464 (M$^+$) and 444 (M – H$^+$)$^+$) and identical retention time to that observed with their corresponding stable isotope-labeled fragment ions were readily observed for the n-propyl-perheptafluorylbutyryl derivative of 3-nitrotyrosine. Moreover, reduction of the products prior to analysis by GC/MS demonstrated ions with identical retention time and appropriate m/z for that of the n-propyl perheptafluorylbutyryl derivative of 3-aminotyrosine (m/z 806 (M – HF)$^+$, m/z 762 (M – HF – CO$_2$)$^+$ and m/z 628 (M – heptafluorylbutyryl)$^+$). Collectively, these results unambiguously confirm that EPO catalyzes nitration of protein tyrosyl residues in the presence of the co-substrates, H$_2$O$_2$ and NO$_2$$_{3}^{-}$.

To characterize the significance of EPO-dependent protein nitration, the content of protein-bound 3-nitrotyrosine produced by the enzyme was determined by reverse phase HPLC with electrochemical detection as described under "Experimental Procedures." BSA (0.4 mg/ml) was incubated with eosinophil peroxidase (57 nM), H$_2$O$_2$ (100 μM), and the indicated concentrations of NO$_2$$_{3}^{-}$ in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. After incubation, 1.2 μg of protein was loaded on 10% SDS-polyacrylamide gels for electrophoresis, and proteins were transferred and immunostained using a rabbit polyclonal antibody against 3-nitrotyrosine (top) or stained with Coomassie Blue (bottom).
peroxide-generating system (glucose/glucose oxidase), and concentrations of halides and NO$_2^-$ as might be observed in inflammatory fluids (i.e. 100 mM Cl$^-$, 100 mM Br$^-$, and 100 mM NO$_2^-$).

Again, EPO nitrated protein tyrosyl residues to a greater extent than that of all other tyrosine oxidation products examined (Table III). Under the conditions employed, only minimal amounts of o,o'-dityrosine and no detectable levels of 3-chloro-tyrosine were formed (Table III). EPO-catalyzed bromination of protein tyrosyl residues occurred, albeit at a level approximately one-third that of tyrosine nitration. Generation of the tyrosine oxidation products demonstrated an absolute requirement for EPO and the H$_2$O$_2$-generating system. Collectively, these results demonstrate that EPO uses NO$_2^-$ as a preferred substrate to form a reactive nitrogen intermediate(s) even in the presence of physiological levels of halides.

The pseudohalide SCN$^-$ is a preferred substrate of EPO (18–20). We were therefore interested in examining the potential influence of SCN$^-$ levels on EPO-catalyzed nitration. Extracellular fluid and tissue levels of this unusual anion have not been reported; however, plasma levels of SCN$^-$ vary considerably (normal values are <69 $\mu$M) depending upon diet and smoking habits in normal individuals (57). In a separate set of experiments, either free L-tyrosine (Fig. 9, left) or BSA (Fig. 9, right) was incubated with NO$_2^-$ (100 $\mu$M) and plasma levels of halides (100 $\mu$M Cl$^-$ and 100 $\mu$M Br$^-$) in the absence and presence of varying amounts of SCN$^-$, and the content of 3-nitrotyrosine was determined. EPO-mediated nitration was attenuated by the addition of SCN$^-$ over the reported range of plasma levels of SCN$^-$ (Fig. 9). In the presence of equivalent levels of NO$_2^-$ and SCN$^-$ (100 $\mu$M each), 3-nitrotyrosine formation was significantly inhibited; however, nitration of both free and protein-bound tyrosine residues was still detectable.

Eosinophil Peroxidase Is More Effective Than Myeloperoxidase at Generating Nitrotyrosine in the Presence of NO$_2^-$, H$_2$O$_2$, and Plasma Levels of Halides—Since MPO-dependent formation of nitrotyrosine may also contribute to protein nitration at sites of inflammation (25–28, 58, 59), we sought to compare the ability of isolated MPO and EPO to nitrate target proteins.
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TABLE III
Oxidation of protein tyrosyl residues by eosinophil peroxidase in the presence of physiological concentrations of Cl\(^-\), Br\(^-\), and NO\(_2\) 

| Reaction mixture | 3-Nitrotyrosine/tyrosine | 3-Bromotyrosine/tyrosine | \(\alpha,\alpha^\prime\)-Dityrosine/tyrosine | 3-Chlorotyrosine/tyrosine |
|------------------|--------------------------|--------------------------|------------------------------------------|--------------------------|
| BSA/EPO/GGOx/NO\(_2\)/Br\(^-\)/Cl\(^-\) | 42.0 ± 5.1 | 13.4 ± 0.9 | <1 | ND\(^b\) |
| Without EPO | ND | ND | ND | ND |
| Without GGOx | ND | ND | ND | ND |
| Without Br\(^-\)/Cl\(^-\)/NO\(_2\) | 54.7 ± 5.3 | ND | <1 | ND |
| Without Br\(^-\)/Cl\(^-\) | 31.0 ± 3.7 | 13.4 ± 1.4 | <1 | ND |
| Without Cl\(^-\) | 62.7 ± 0.5 | ND | ND | ND |

\(^a\) GGOx, glucose/glucose oxidase. 
\(^b\) ND, not detected.

BSA (0.4 mg/ml) was incubated with eosinophil peroxidase (57 nM), glucose (100 μg/ml), glucose oxidase (20 μg/ml), and the indicated anionic substrates (Br\(^-\) = NO\(_2\) = 100 μM, Cl\(^-\) = 100 mM) in sodium phosphate buffer (20 mM, pH 7.0) at 37°C for 4 h. Following reaction, protein was desalted and subjected to acid hydrolysis, and the content of 3-nitrotyrosine, 3-bromotyrosine, \(\alpha,\alpha^\prime\)-dityrosine, and 3-chlorotyrosine was determined by reverse phase HPLC with electrochemical detection as described under “Experimental Procedures.” Results are the mean ± S.D. of three independent experiments. The limit of detection for these analyses is <0.05 mmol/mol tyrosine for each analyte examined.

![Graph](image)

**Fig. 9.** Inhibitory effect of SCN\(^-\) on 3-nitrotyrosine production by eosinophil peroxidase. Left, L-tyrosine (100 μM) was incubated with eosinophil peroxidase (57 nM), H\(_2\)O\(_2\) (100 μM), and NO\(_2\) (100 μM) in the presence of the indicated concentrations of NaSCN in sodium phosphate buffer (20 mM, pH 7.0) at 37°C for 1 h. The content of 3-nitrotyrosine formed was then determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of results from three independent experiments. Right, BSA (0.4 mg/ml) was incubated with eosinophil peroxidase (57 nM), glucose (100 μg/ml), glucose oxidase (20 μg/ml), and NO\(_2\) (100 μM) in the presence of the indicated concentrations of NaSCN in sodium phosphate buffer (20 mM, pH 7.0) at 37°C overnight. The content of protein-bound nitrotyrosine formed was then determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of results from three independent experiments.

BSA was incubated with equal concentrations of each peroxidase in the presence of plasma levels of halides (100 mM Cl\(^-\) and 100 μM Br\(^-\)) and an H\(_2\)O\(_2\)-generating system (glucose/glucose oxidase) to more closely mimic the low steady flux of reactive nitrogen species such as \(\mathrm{NO}_2\), the one electron oxidation product of NO\(_2\). In the absence of NO\(_2\), incubation of \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine with EPO and H\(_2\)O\(_2\) resulted in formation of the \(\mathrm{N}^\alpha\)-acetyl dityrosine analog (Table IV). The addition of Br\(^-\) to the reaction mixtures resulted in production of the \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analogs of 3-bromotyrosine and 3,5-dibromotyrosine but no detectable production of the \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analog of the tyrosyl radical-addition product, \(\alpha,\alpha^\prime\)-dityrosine. In contrast, the addition of NO\(_2\) to \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine and the EPO-H\(_2\)O\(_2\) system formed predominantly the \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analog of 3-nitrotyrosine and modest levels of the \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analog of \(\alpha,\alpha^\prime\)-dityrosine (Table IV). The addition of both NO\(_2\) and Br\(^-\) to \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine and the EPO-H\(_2\)O\(_2\) system formed all of the expected tyrosine oxidation products (\(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analogs of 3-nitrotyrosine, \(\alpha,\alpha^\prime\)-dityrosine, 3-bromotyrosine, and 3,5-dibromotyrosine). Thus, in every instance where tyrosine nitration occurred, concurrent formation of tyrosyl radical addition products was observed. In contrast, during tyrosine bromination (in the absence of NO\(_2\)), no formation of tyrosyl radical addition products (i.e., \(\mathrm{N}^\alpha\)-acetyl-L-tyrosine analog of \(\alpha,\alpha^\prime\)-dityrosine) could be detected. These results are consistent with 3-nitrotyrosine production through an addition reaction between an intermediate tyrosyl radical and reactive nitrogen species such as \(\mathrm{NO}_2\), the one electron oxidation product of NO\(_2\).

Co-incubation of HOC\(_{1}\) and NO\(_2\) has been reported to form a nitrating and chlorinating intermediate, presumably NO\(_2\)Cl (58). Moreover, we observed modest increases in the levels of free and protein-bound nitrotyrosine formed during incubation with the EPO-H\(_2\)O\(_2\) system in the presence of NO\(_2\) and Cl\(^-\) compared with NO\(_2\) alone (Tables II and III). However, the mechanism for the increase does not appear to involve reaction of HOC\(_{1}\) with NO\(_2\), since comparable increases in tyrosine nitration were observed in the presence of Cl\(^-\) during incubations that contained excess methionine, a potent scavenger of HOC\(_{1}\) and other halogenating intermediates (data not shown). To determine if EPO might indirectly promote protein nitration by forming a nitrating intermediate by oxidation of NO\(_2\) with HOBr, we performed the following experiment. \(\mathrm{N}^\alpha\)-Acetyl-L-tyrosine was incubated with HOBr in the presence and absence of NO\(_2\), and the extent of tyrosine nitration was determined. No detectable formation of the 3-nitrotyrosine analog was observed, even in the presence of 1 mM concentrations of both HOBr and NO\(_2\) (Table IV). Similar results were observed in reactions performed under acidic (pH 4) conditions (data not shown). Collectively, these results suggest that the nitrating intermediate formed by EPO arises from direct oxidation of NO\(_2\) and not by secondary oxidation of NO\(_2\) by HOCl, HOBr, or some other halogenating agent.

In a final series of experiments, we sought to further explore
the potential role of a tyrosyl radical intermediate in 3-nitrotyrosine formation by monitoring the extent of aromatic nitration using tyrosine analogs that do not form tyrosyl radicals. Because of its acidic character, the phenoxyl hydrogen of L-tyrosine is preferentially abstracted from tyrosine; consequently, tyrosine is the preferred site of hydrogen atom abstraction from aromatic amino acids (60). If 3-nitrotyrosine production by eosinophils is mediated by an electrophilic addition reaction (e.g. through a NO$_2^-$ intermediate), use of the O-methyl-L-tyrosine analog should not significantly affect the yield of 3-nitrotyrosine formation. Incubation of the complete EPO-H$_2$O$_2$-NO$_2^-$ system with L-tyrosine readily formed 3-nitrotyrosine; in contrast, no nitrated products were formed from O-methyl-L-tyrosine at all concentrations of NO$_2^-$ examined, as determined by reverse phase HPLC with on-line UV and ESI/MS (Fig. 11). Collectively, these results strongly support the hypothesis that the EPO-H$_2$O$_2$-NO$_2^-$ system nitrates tyrosyl residues through a tyrosyl radical intermediate.

**DISCUSSION**

Eosinophils play an essential role in tissue surveillance and host defense mechanisms (1–3). These cells have evolved enzymatic mechanisms to inflict oxidative damage upon invading parasites, pathogens, and cancer cells. The reactive species they form, however, also have the potential to harm host tissues and contribute to inflammatory tissue injury. Microbicidal and cytotoxic oxidants generated by the EPO-H$_2$O$_2$ system are thought to participate in promoting many of these functions (2–8). However, despite the numerous links between EPO, oxidant production, and tissue injury in eosinophil inflammatory disorders, structural identification of oxidation products formed by the action of EPO on target proteins is lacking.
Nitrotyrosine Formation by Eosinophil Peroxidase

One of the remarkable features of protein nitration by the EPO-H$_2$O$_2$-NO$_2^-$ system is the overall high yield of 3-nitrotyrosine formation. In the presence of plasma levels of halides and levels of NO$_2^-$ that approximate those found in inflammatory tissues and fluids, 3-nitrotyrosine was a preferred product formed with both free and protein-bound tyrosine residues as targets (e.g., Tables II and III). Moreover, tyrosine nitration was optimal in a physiological pH range (6.0–7.0) and accounted for between 10 and 20% of the H$_2$O$_2$ consumed during oxidation of proteins by the EPO-H$_2$O$_2$-NO$_2^-$ system at neutral pH. Finally, common nucleophilic scavengers such as primary amines, thiols, and thiol ethers only modestly attenuated nitrotyrosine formation; however, physiological levels of glutathione, ascorbate, and SCN$^-$ are anticipated to have a major impact on the extent of nitrotyrosine formation by EPO. Taken together, these results suggest that EPO-dependent nitration of protein tyrosyl residues is likely to occur in vivo, particularly at sites where eosinophil infiltration and NO (and hence NO$_2^-$) production are enhanced and ascorbate, glutathione, and SCN$^-$ levels are limiting. Indeed, recent immunohistochemical studies using antibodies raised against nitrotyrosine demonstrated intense staining over eosinophils and throughout eosinophil-rich tissues of individuals with severe asthma (22), a condition where eosinophils are implicated in promoting inflammatory tissue injury and enhanced oxidant stress and NO production occur (2–6, 61–64). Although the authors interpreted the 3-nitrotyrosine immunostaining as evidence of peroxynitrous acid production (22), the current results suggest that an alternative source may be the EPO-H$_2$O$_2$-NO$_2^-$ system of eosinophils.

It is interesting to note that the extent of 3-nitrotyrosine formation by EPO is modulated by physiologically relevant levels of SCN$^-$. Thiocyanate ion is produced following the enzymatic hydrolysis of certain plant glycosides. Thus, eating certain vegetables (e.g., cabbage) results in a transient increase in the concentration of thiocyanate in blood and urine (57). Normal plasma levels of SCN$^-$ are under 70 $\mu$M (57), but higher levels may be observed following excessive tobacco smoking or prolonged intravenous administration of sodium nitroprusside, a potent vasodilator used in critically ill patients (57, 65). Eosinophils perform their biological functions in a large variety of tissues and fluids whose concentration of halides, NO$_2^-$, and SCN$^-$ may differ considerably from plasma. Indeed, our kinetic studies (Table I) suggest that at plasma concentrations of substrates (SCN$^-$, Br$^-$, NO$_2^-$, and Cl$^-$), EPO is far from saturated. Thus, the relative contribution of EPO to 3-nitrotyrosine formation in vivo may be tissue-specific, even at sites of inflammation characterized by eosinophil recruitment and activation.

The formation of reactive nitrogen species by direct peroxidase-dependent oxidation of NO$_2^-$, or through oxidation of NO$_2^-$ by oxidants such as HOCI, has recently been reported (25–28, 58, 59). Indeed, the ability of MPO secreted from activated neutrophils to catalyze aromatic nitration reactions has suggested that nitrotyrosine formation at sites of inflammation may arise from pathways independent of peroxynitrous acid generation (26). However, whether EPO could catalyze similar reactions given the unusual substrate preferences, distinct prosthetic heme group, and unique physical properties of this highly cationic protein was unclear. The results of the present studies demonstrate that aromatic nitration reactions are indeed a preferred activity of the enzyme. Moreover, the nitrating capacity of EPO was shown to exceed that of the related leukocyte-derived peroxidase, MPO, by at least 4-fold at physiological concentrations of halides and every concentration of NO$_2^-$ examined. Finally, it should be noted that O$_2^-$ and H$_2$O$_2$ production by activated eosinophils is severalfold greater than...
that of an equal number of comparably stimulated neutrophils (9, 10) and that the total content of EPO in eosinophils is reported to be 2–4-fold higher than that of MPO in neutrophils (e.g. 30–33 pmol of MPO per 1 x 10⁶ neutrophils versus 70–135 pmol of EPO per 1 x 10⁶ eosinophils) (16, 66, 67). Thus, EPO-dependent formation of reactive nitrogen intermediates is likely to occur in vivo, particularly during inflammatory disorders characterized by the presence of eosinophils.

Another interesting distinction between EPO- and MPO-dependent nitration reactions arises from their differing halide specificity. Although MPO generates chlorinating oxidants at physiological concentrations of halides (46, 68–70), and HOCl is reported to react with NO₂⁻ to form a nitrating intermediate (58), we found no evidence for a parallel reaction between NO₂⁻ and HOB₃⁻, the primary halogenating intermediate formed by EPO at plasma levels of halides. Moreover, although EPO-dependent nitration of tyrosine was optimal in the presence of both NO₂⁻ and plasma levels of Cl⁻ (e.g. see Tables II and III), the addition of methionine, a potent scavenger of halogenating intermediates (46, 71, 72), failed to attenuate nitrotyrosine formation by EPO. The mechanism of the modest but reproducible increase in both free and protein-bound 3-nitrotyrosine formation observed in the presence versus absence of Cl⁻ is unclear. The inability of methionine to attenuate tyrosine nitration by EPO in the presence of either Br⁻ or Cl⁻ suggests that indirect oxidation of NO₂⁻ by an EPO-generated hypohalous acid does not contribute significantly to tyrosine nitration.

One interesting question is the chemical nature of the reactive nitrogen species formed by EPO-catalyzed oxidation of NO₂⁻. Peroxidases catalyze both one- and two-electron oxidation reactions, and both the one-electron (i.e. nitrogen dioxide) and two-electron (i.e. peroxynitrite) oxidation products of NO₂⁻ have been suggested as potential intermediates formed by MPO (25, 28). Both intermediates can form nitrotyrosine, as well as abstract a phenoxyl hydrogen atom to form tyrosyl radicals. Intermediate. Future studies aimed at elaborating the oxidant(s) formed and the mechanisms of free and protein-bound tyrosine nitration mediated by peroxidase-dependent oxidation of NO₂⁻ are warranted. Eosinophils are typically rare leukocytes with a limited circulating life span (t½ ~ 2 h) before they take residence in tissues (1). However, they do play a prominent role in certain forms of inflammation (e.g. allergic), where their numbers in tissues can greatly exceed those of other leukocytes (1–6). Indeed, some of the most devastating inflammatory disorders in humans are associated with intense eosinophilic infiltration (1–6, 73, 74). Identifying the mechanisms and products of EPO-dependent oxidative damage is a critical step toward development of targeted interventions designed to interrupt oxidative tissue injury in eosinophilic inflammatory disorders.
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