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J Immunol 2001; 166:5763-5772; ;
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The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Eosinophils Are a Major Source of Nitric Oxide-Derived Oxidants in Severe Asthma: Characterization of Pathways Available to Eosinophils for Generating Reactive Nitrogen Species¹

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Eosinophil recruitment and enhanced production of NO are characteristic features of asthma. However, neither the ability of eosinophils to generate NO-derived oxidants nor their role in nitration of targets during asthma is established. Using gas chromatography-mass spectrometry we demonstrate a 10-fold increase in 3-nitrotyrosine (NO₂Y) content, a global marker of protein modification by reactive nitrogen species, in proteins recovered from bronchoalveolar lavage of severe asthmatic patients (480 ± 198 μmol/mol tyrosine; n = 11) compared with nonasthmatic subjects (52.5 ± 40.7 μmol/mol tyrosine; n = 12). Parallel gas chromatography-mass spectrometry analyses of bronchoalveolar lavage proteins for 3-bromotyrosine (BrY) and 3-chlorotyrosine (ClY), selective markers of eosinophil peroxidase (EPO)- and myeloperoxidase-catalyzed oxidation, respectively, demonstrated a dramatic preferential formation of BrY in asthmatic (1093 ± 457 μmol BrY/mol tyrosine; 161 ± 88 μmol ClY/mol tyrosine; n = 11 each) compared with nonasthmatic subjects (13 ± 14.5 μmol BrY/mol tyrosine; 65 ± 69 μmol ClY/mol tyrosine; n = 12 each). Bronchial tissue from individuals who died of asthma demonstrated the most intense anti-NO₂Y immunostaining in epitopes that colocalized with eosinophils. Although eosinophils from normal subjects failed to generate detectable levels of NO, NO₂⁻, NO₃⁻, or NO₂Y, tyrosine nitration was promoted by eosinophils activated either in the presence of physiological levels of NO₂⁻ or an exogenous NO source. At low, but not high (e.g., >2 μM/min), rates of NO flux, EPO inhibitors and catalase markedly attenuated aromatic nitration. These results identify eosinophils as a major source of oxidants during asthma. They also demonstrate that eosinophils use distinct mechanisms for generating NO-derived oxidants and identify EPO as an enzymatic source of nitrating intermediates in eosinophils. *The Journal of Immunology*, 2001, 166: 5763–5772.

Eosinophils play a specialized role in innate host defense. These granulocytes are recruited to tissues to combat invading pathogens, such as during parasitic and helminthic infections (1–5). However, eosinophil recruitment is also a characteristic feature of asthma and other allergic inflammatory disorders (3–7). Although the function of eosinophils during host defense scenarios seems apparent, whether they play a beneficial role in allergy and asthma is less certain. Rather, substantial evidence supports the notion that eosinophils contribute to tissue injury and many of the pathophysiological features of asthma (3–7). Clinically, elevated eosinophil counts and the presence of eosinophil-secreted granular proteins such as major basic protein, eosinophil

cationic protein, or eosinophil peroxidase (EPO)³ in sputum and biopsy samples have been used as biomarkers for monitoring the severity of asthmatic events (3–10). Though high levels of granular proteins are indicative of eosinophilia, leukocyte activation, and degranulation, little is known about the precise chemical mechanisms through which eosinophils contribute to tissue damage in the asthmatic lung and airways.

Eosinophils are unique among circulating leukocytes in their prodigious capacity to wage chemical warfare. As mentioned above, they are endowed with numerous highly basic and cytotoxic granule proteins that are released upon activation or during cell necrosis. They also possess an arsenal of enzymes designed to inflict oxidative damage upon biological targets (1–3). During eosinophil activation, a respiratory burst occurs where O₂^{•-} and its dismutation product, H₂O₂, are formed (1, 2, 11). The respiratory burst of human eosinophils produces anywhere from 3 to 10 times as much O₂^{•-} as a corresponding number of similarly treated neutrophils (12, 13). Although reduced oxygen species such as O₂^{•-} and H₂O₂ do not effectively oxidize biological targets, one of the most abundant proteins secreted upon eosinophil activation, EPO, amplifies the oxidizing potential of H₂O₂ by using it as cosubstrate

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Received for publication October 6, 2000. Accepted for publication February 22, 2001.

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¹ This work was supported by National Institutes of Health Grants HL61878 and HL62526 and by the American Heart Association. J.M. was supported by a National Institutes of Health NRSA Fellowship.

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³ Abbreviations used in this paper: EPO, eosinophil peroxidase; BAL, bronchoalveolar lavage; Br-HPA, 3-(3-bromo-4-hydroxyphenyl)propanoic acid; BrY, 3-bromotyrosine; Cl-HPA, 3-(3-chloro-4-hydroxyphenyl)propanoic acid; ClY, 3-chlorotyrosine; DTPA, diethylenetriaminepentaacetic acid; GC-MS, gas chromatography-mass spectrometry; hi-Cat, heat inactivated catalase; HPA, 3-(4-hydroxyphenyl) propanoic acid; NO₂-HPA, 3-(4-hydroxy-3-nitrophenyl)propanoic acid; NO₂Y, 3-nitrotyrosine; PAPA NONOate, (Z)-[N-(3-aminopropyl)-N-(n-propyl)amino]diazene-1-ium-1,2-diolate; RNS, reactive nitrogen species; SOD, superoxide dismutase.

to generate potent reactive oxidants and diffusible radical species (2, 14–16).

EPO is a member of the mammalian peroxidase superfamily (17) and has a high degree of sequence homology with myeloperoxidase (MPO), an abundant peroxidase in neutrophils, monocytes, and certain tissue macrophages (18). Both EPO and MPO share the unique ability to use halides and pseudohalides as substrates to make highly reactive oxidants, hypohalous acids (HOX) (Equation 1).



where $\text{X} = \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{or } \text{SCN}^-$.

At normal plasma levels of halides and pseudohalides (100 mM Cl^- , 20–150 μM Br^- , <69 μM SCN^- , 0.1–0.6 μM I^- ; Ref. 19), EPO preferentially uses the pseudohalide SCN^- (20) and Br^- as substrates (15, 21), whereas MPO uses Cl^- (22, 23). Recent gas chromatography-mass spectrometry (GC-MS) studies demonstrate that eosinophils use EPO to generate oxidants in allergen-triggered asthma (24). BrY (3-bromotyrosine), a specific marker of protein modification by reactive brominating species (25), was markedly enriched in bronchoalveolar lavage (BAL) proteins recovered from asthmatic subjects following exposure to segmental allergen challenge (24). Thus, one chemical pathway used by eosinophils to promote oxidative modification of proteins during asthma is through EPO-generated reactive brominating species.

Another potential pathway for oxidative modification of tissues in asthma by eosinophils may involve formation of reactive nitrogen species (RNS). NO production is increased in asthma (26–28). Although NO is a relatively long-lived radical that does not nitrate biological targets directly, it can be converted into more potent RNS. The most commonly studied NO-derived oxidant is peroxynitrite (ONOO^-), a product formed by the near diffusion-limited interaction of NO and $\text{O}_2^{\cdot-}$ (29). ONOO^- nitrates protein tyrosine residues (29–31), and immunohistochemical studies suggest that 3-nitrotyrosine (NO_2Y) is produced in asthmatic airways (32, 33). At physiological levels of $\text{CO}_2/\text{HCO}_3^-$, ONOO^- exists predominantly as the more potent nitrating species, peroxycarboxynitrite (ONOOCO_2^-) (Refs. 34–36; Equation 2).



Concomitant production of NO and $\text{O}_2^{\cdot-}$ almost invariably occurs at sites of inflammation; consequently, potential adverse effects of excess NO production have been primarily attributed to formation of ONOO^- or ONOOCO_2^- (29, 32, 33, 36, 37).

However, recent studies have identified alternative pathways for generating NO-derived oxidants that involve leukocyte peroxidases. van der Vliet and colleagues first demonstrated that MPO could use nitrite (NO_2^-), a major end-product of NO metabolism, as a substrate to nitrate tyrosine residues (38). Subsequent studies with isolated neutrophils (39, 40) and monocytes (41–43) have demonstrated that MPO-generated RNS can play a significant, and even predominant, role in oxidative modification of biomolecules. We recently demonstrated that EPO is significantly more effective than MPO at promoting protein nitration at physiologically relevant concentrations of halides and NO_2^- (44). Although increased levels of NO, NO_2^- , H_2O_2 , and eosinophils are present in asthmatic airways, neither the ability of eosinophils to generate NO-derived oxidants nor their role in nitration of targets during asthma are reported.

In this study we demonstrate that RNS play a significant role in oxidative modification of proteins in asthma. Using a combination of approaches including mass spectrometry, immunohistochemistry, and studies with isolated eosinophils, we show that eosinophils generate NO-derived oxidants both in vitro and in vivo. Finally,

we examine the chemical pathways available to human eosinophils for generating NO-derived oxidants and demonstrate that the leukocytes form RNS by at least two mechanistically distinct pathways.

Materials and Methods

General materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and were Optima or HPLC grade. PAPA NONOate (Z)-[N-(3-aminopropyl)-N-(n-propyl)amino]diazene-1-ium-1,2-diolate was obtained from Alexis (San Diego, CA). Glucose oxidase (grade II) and catalase were acquired from Roche Molecular Biochemicals (Indianapolis, IN). L-[$^{13}\text{C}_6$, ^{15}N] tyrosine and L-[$^{13}\text{C}_6$] tyrosine were obtained from Cambridge Isotope Laboratories (Andover, MA). Unless otherwise stated, all other chemicals were purchased from Sigma (St. Louis, MO).

Subjects and sample collection

Human endotracheal/bronchial aspirates were obtained from patients ($n = 11$) who required mechanical ventilatory assistance due to respiratory failure from a severe asthmatic exacerbation. All asthmatic subjects had a history of a >12% increase in absolute forced expiratory volume in 1 s (FEV_1) and a 200-cc increase in forced expiratory volume either spontaneously or after bronchodilator within 1 year of hospitalization, and satisfied the definition of severe asthma as defined by the National Institutes of Health guidelines (45). Samples were collected within 12 h of admission because asthmatic subjects received i.v. corticosteroids upon presentation to the emergency department and subsequent transfer to the intensive care unit, and the effects of corticosteroids on protein oxidation products is unknown. All asthmatic individuals had a history of using inhaled β_2 -agonists either regularly ($n = 8$) or on an as-needed basis ($n = 3$) in the month preceding admission. Several also used inhaled corticosteroids ($n = 8$) and/or had received oral corticosteroids within 1 month of presentation ($n = 4$). Control subjects ($n = 12$) were age- and sex-matched, nonsmokers, and had no prior history of asthma or other lung disease. Endotracheal/bronchial aspirates were obtained from several control subjects who were either undergoing elective surgery ($n = 3$) or were admitted to the intensive care unit for a noninflammatory, nonrespiratory process (i.e., airway protection secondary to either head trauma ($n = 2$) or drug overdose ($n = 2$)). The remaining clinical specimens from healthy nonasthmatic controls ($n = 5$) were obtained as residual specimens collected as baseline BAL samples for a separate clinical study (46). Healthy control subjects in that study all had a negative methacholine challenge test. Thus, all 11 specimens from asthmatic subjects were obtained as endotracheal/bronchial aspirates, and the 12 specimens from nonasthmatic controls were obtained as endotracheal/bronchial aspirates ($n = 7$) and baseline BAL ($n = 5$) specimens. No differences were noted in levels of NO_2Y , BrY, or 3-chlorotyrosine (ClY) (per mol tyrosine) in endotracheal/bronchial aspirates vs BAL; consequently, specimens from the nonasthmatic subjects were combined as a single control group. Cells in clinical specimens were removed by centrifugation, the supernatants were supplemented with a mixture of antioxidants and peroxidase inhibitors (final concentration: 200 μM diethylenetriamine-pentaacetic acid (DTPA), 100 μM butylated hydroxytoluene, 50 mM sodium phosphate (pH 7.0), 1 mM aminotriazole), capped in vials under argon atmosphere, snap frozen in liquid N_2 , and stored at -80°C until time of sample preparation and mass spectrometry analysis.

Histological analysis of lung and bronchial tissues

Histological sections were cut from paraffin blocks of lung and bronchial tissues from four individuals who died from asthma and four nonasthmatic age-matched individuals who died of nonpulmonary processes. For NO_2Y immunostaining, slides were initially incubated at 37°C with 0.01 mg/ml protease K for 15 min. Following wash with PBS containing 0.5 mM levamisole, the tissue was treated with 1% BSA in PBS to block nonspecific binding, then incubated for 2 h with immunopurified polyclonal Ab directed against NO_2Y (1:150 diluted in 1% BSA/PBS; Upstate Biotechnology, Lake Placid, NY). Following wash with PBS/0.5 mM levamisole, the tissue was incubated with a biotin-conjugated secondary Ab (Dako, Carpinteria, CA) for 10 min. Washing was followed by another 10-min incubation with alkaline phosphatase-labeled streptavidin (Dako). Immunostaining was visualized with an alkaline phosphate substrate solution containing naphthol AS-MX phosphatase, Fast Red, and levamisole in Tris buffer (pH 8.2) (Dako) and counterstained with the nuclear stain, hematoxylin. Negative control experiments involved either immunoadsorption of NO_2Y Ab with 3.75 mM NO_2Y before incubation with tissue sections or incubating the section with isotype-control nonimmune Ig instead of the

primary Ab. In separate studies, the specificity of the primary Ab was confirmed by observing loss of nitrotyrosine-specific recognition following reduction of nitrotyrosine-containing protein with dithionite. All biopsies were stained on the same day. Specific autofluorescence imaging of EPO in biopsies was performed as described (47–49). EPO-specific in situ peroxidase staining of tissues was performed on anti-nitrotyrosine-stained tissue sections following treatment of slides with 0.01 M KCN to inhibit MPO (50, 51).

Eosinophil and neutrophil isolation

Human eosinophils were isolated by negative selection using CD16 microbeads (Miltenyi Biotec, Auburn, CA), as described (52). Human neutrophils were isolated by buoyant density centrifugation (53), and low levels of contaminating eosinophils were then removed by fluorescence-activated cell sorting (54). The final purity of cell preparations was confirmed by flow cytometry using selective Abs for cell surface Ag on eosinophils (CD49d) and neutrophils (CD16), respectively (54). No detectable cross-contamination of peroxidase activity in detergent extracts of leukocyte preparations was observed following SDS-PAGE with in-gel tetramethylbenzidine peroxidase staining (55). Trypan blue exclusion tests demonstrated over 97% viability in eosinophil and neutrophil preparations.

Cell experiments

These studies were performed in the presence of CO₂ (5% gas phase) and HCO₃⁻ in the medium (4.2 mM) to more closely mimic a biologically relevant situation. Leukocytes (1 × 10⁶/ml) were incubated at 37°C under 95% air, 5% CO₂, in Medium A (Ca²⁺/Mg²⁺/phenol-free HBSS; Life Technologies, Gaithersburg, MD) supplemented with 100 μM NaBr, 50 μM L-arginine, and 200 μM DTPA, pH 7.4 final) in the absence or presence of NaNO₂ (0–50 μM, as indicated in the figure legends). Where indicated, 100 μM of either L-tyrosine or its deaminated analog, 3-(4-hydroxyphenyl)propanoic acid (HPA) was included. Cells were activated by addition of PMA (200 nM) and incubated for either 1 h or the indicated time interval. In some experiments, eosinophils were activated in the presence of an exogenous NO source by addition of PAPA NONOate (Alexis) for 1 min following PMA addition. Rates of NO flux were determined spectrophotometrically by reaction of NO with oxyhemoglobin (56) under the identical conditions used for experiments, but in the absence of any added cells. To maintain a final pH of 7.4 during experiments with PAPA NONOate, incubations were performed in Medium B (Medium A containing only 100 mM NaCl and supplemented with 20 mM sodium phosphate, pH 7.4). NO₂⁻ levels accumulated to over 50 μM in medium incubated with the NO-generating system (2 μM/min) in the absence of cells. In some cases, leukocyte reaction mixtures also contained one of the following: 1 mM NaN₃, 10 mM 3-aminotriazole (Atz), 300 nM catalase (Cat), 300 nM heat-inactivated catalase (hiCat), 10 μg/ml superoxide dismutase (SOD), 1 mM methionine (Met), or 1 mM N^ε-acetyl lysine.

Quantification of leukocyte-generated products in vitro

NO₂Y production by isolated eosinophils was quantified by reversed phase HPLC with photodiode array detection (44). Peak identity was routinely confirmed by demonstrating the appropriate UV-VIS absorbance spectrum of the peak that comigrated with authentic NO₂Y. In preliminary studies, NO₂Y production by eosinophils was also independently confirmed by HPLC with on-line electrospray ionization mass spectrometry, similar to prior studies using isolated EPO (44). The nitrated (NO₂-HPA; 3-(4-hydroxy-3-nitrophenyl)propanoic acid), brominated (Br-HPA; 3-(3-bromo-4-hydroxyphenyl)propanoic acid), and chlorinated (Cl-HPA; 3-(3-chloro-4-hydroxyphenyl)propanoic acid) products of the tyrosine analog, HPA, were routinely quantified by reversed phase HPLC with electrochemical (coulometric) detection on an ESA CoulArray HPLC (Cambridge, MA) equipped with UV detector and electrochemical cells (eight channels) (44). Peak identity was established by demonstrating the appropriate retention time, redox potential, ratio of integrated currents in adjacent channels, and by the method of standard additions for each analyte. Authentic standards of NO₂-HPA, Br-HPA, and Cl-HPA were prepared by reaction of HPA with a molar equivalent of ONOO⁻, HOBr, or HOCl, respectively. Standards were then isolated by reversed phase HPLC, and their structures were confirmed by electrospray ionization mass spectrometry by demonstrating the appropriate mass-to-charge ratio (and isotopic cluster, where applicable) of the anticipated molecular ion of the isolated product.

Sample preparation and mass spectrometry

The contents of BrY and ClY in proteins present in clinical specimens were determined by stable isotope dilution GC-MS using 3-bromo[¹³C₆]tyrosine and 3-chloro[¹³C₆]tyrosine as internal standards (24). The NO₂Y content of

lavage proteins was quantified by stable isotope dilution GC-MS following reduction to aminotyrosine (57). All results were normalized to the content of the precursor amino acid, L-tyrosine, which was similarly quantified by GC-MS (58) using L-[¹³C₆,¹⁵N]tyrosine as internal standard. Intrapreparative formation of 3-bromo[¹³C₆,¹⁵N]tyrosine, 3-chloro[¹³C₆,¹⁵N]tyrosine, or 3-nitro[¹³C₆,¹⁵N]tyrosine was routinely monitored and found to be negligible (i.e., <5% of the level of the natural abundance product observed) under the conditions used.

General procedures

All water used to prepare buffers and medium was pretreated with Chelex-100 resin (Bio-Rad, Hercules, CA) and supplemented with 100 μM DTPA to remove trace levels of potential redox-active transition metal ions. Superoxide generation by activated human eosinophils was measured as the SOD-inhibitable reduction of ferricytochrome *c* (59). Quantification of NO₂⁻ and NO₃⁻ was performed by anion exchange HPLC with UV detection at 210 nm under argon atmosphere. Products were resolved on a Spherisorb S5 SAX column (24 cm × 4.6 mm, 5 μm; Phase Separations, Norwalk, CT) under isocratic conditions using 45 mM sodium phosphate (pH 3.0) as the mobile phase.

Leukocytes were isolated from whole blood of healthy volunteers after obtaining informed consent. Tissue sections were obtained from the New Mexico Office of the Medical Examiner and the Anatomic Pathology Department at the Cleveland Clinic Foundation. Sections were anonymized by the forensic pathologists for use in these studies. All protocols were in accordance with institutional guidelines of either the University of New Mexico School of Medicine or the Cleveland Clinic Foundation and were approved by their respective Institutional Review Committees.

Statistics

Data represent the mean ± SD of the indicated number of samples. Statistical analyses were made using a paired Student's *t* test. For all hypotheses the significance level was 0.05. When multiple comparisons were made, a Bonferroni correction to the significance criterion for each test was made.

Results

RNS contribute to oxidative modification of proteins in asthma

To quantify the potential role of RNS in promoting protein oxidation in asthma, we used stable isotope dilution GC-MS to compare the protein content of NO₂Y recovered from airways of subjects with severe asthma vs nonasthmatic subjects. A significant

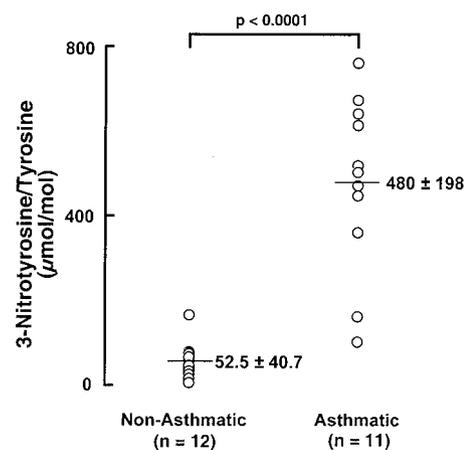


FIGURE 1. Stable isotope dilution GC-MS analysis of NO₂Y in proteins recovered in endotracheal/bronchial aspirates from severe asthmatic and nonasthmatic subjects. The content of NO₂Y in proteins recovered from endotracheal/bronchial aspirates was measured by stable isotope dilution GC-MS in negative ion chemical ionization mode as described in *Materials and Methods*. Data points represent the mean of duplicate determinations of samples from each individual. Numbers in parentheses represent the number of subjects in each group. Mean values ± SD for each group are shown. The levels of nitrotyrosine were substantially higher for asthmatic vs nonasthmatic subjects ($p < 0.0001$).

($p < 0.0001$) 10-fold increase in protein NO₂Y levels was observed in samples from severe asthmatic patients compared with levels present in nonasthmatic subjects (480 ± 198 vs 52.5 ± 40.7 $\mu\text{mol/mol}$ tyrosine; asthmatic vs nonasthmatic, respectively; Fig. 1).

Immunohistochemical studies colocalize nitrotyrosine with eosinophils in bronchial tissues from severe asthmatics

To identify the potential cellular source(s) of NO-derived oxidants in severe asthma, specimens from subjects who died from asthma (*status asthmaticus*) were examined using affinity-purified Ab specific for NO₂Y. Intense staining that colocalized with eosinophils was typically observed in the majority of specimens (Fig. 2). Diffuse staining of epithelial cells was also commonly observed, as has previously been reported (32, 33). Both in situ fluorescence

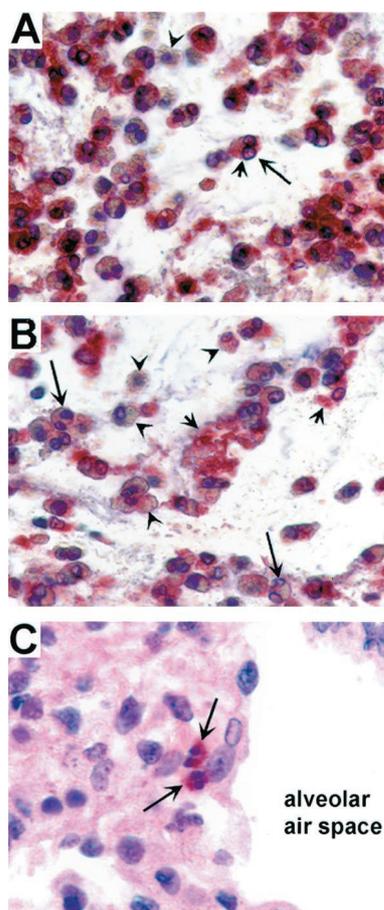


FIGURE 2. Colocalization of nitrotyrosine immunohistochemical staining and eosinophil peroxidase-specific cytochemical staining in bronchial tissues from individuals with *status asthmaticus*. Lung and bronchial tissue from individuals (A, B, and C) who died of asthma were sequentially immunostained for NO₂Y followed by cytochemical staining for EPO. A and B, Colocalization of EPO and NO₂Y in the submucosa of bronchial tissues from distinct subjects with *status asthmaticus*. Intense eosinophilia and NO₂Y staining are prominent in these specimens. The EPO is present as pale golden brown granules (arrow head) within intact eosinophils (some of which have characteristic bilobed nuclei; long arrows) and fragments of eosinophils. The NO₂Y stains bright red (short arrows) and is present as extracellular granular material or within the cytoplasm of some of the eosinophils. C, In this subject, eosinophils and NO₂Y staining were much less frequent. Intense NO₂Y staining is predominantly observed colocalizing within intact eosinophils, which are recognizable by their characteristic bilobed nuclei. The strong red staining for NO₂Y obscured the cytoplasmic staining for EPO in these eosinophils. Original magnification $\times 500$ for all photomicrographs. Hematoxylin counterstain.

microscopy specific for the heme group of EPO (data not shown) and in situ peroxidase staining specific for EPO were also abundant in eosinophil-rich areas of specimens from asthmatics. Double staining of sections for both NO₂Y- and EPO-specific in situ peroxidase staining confirmed colocalization of NO₂Y with eosinophils in the submucosa of airways from severe asthmatic subjects (Fig. 2).

Eosinophils are a major cellular mediator of protein oxidation in severe asthma

To assess the relative contributions of eosinophils and neutrophils in the oxidative modification of proteins in severe asthma, the protein content of BrY and ClY, molecular fingerprints for eosinophil- and neutrophil-mediated tissue damage, respectively (24, 60), were determined in the same clinical specimens evaluated for protein NO₂Y content in Fig. 1. There was a striking 84-fold elevation ($p < 0.0001$) in the content of BrY observed in proteins recovered from airways of asthmatic (1093 ± 457 $\mu\text{mol BrY/mol}$ tyrosine) vs nonasthmatic subjects, whose levels were near the limit of detection (13 ± 14.5 $\mu\text{mol BrY/mol}$ tyrosine) (Fig. 3A). There was also a significant 3-fold increase ($p < 0.05$) in ClY in airway proteins recovered from severe asthmatics (161 ± 88 $\mu\text{mol ClY/mol}$ tyrosine) over nonasthmatics (65 ± 69 $\mu\text{mol ClY/mol}$ tyrosine) (Fig. 3B). A comparison of the BrY/ClY ratios, an indication of the relative preferential contribution of eosinophils vs

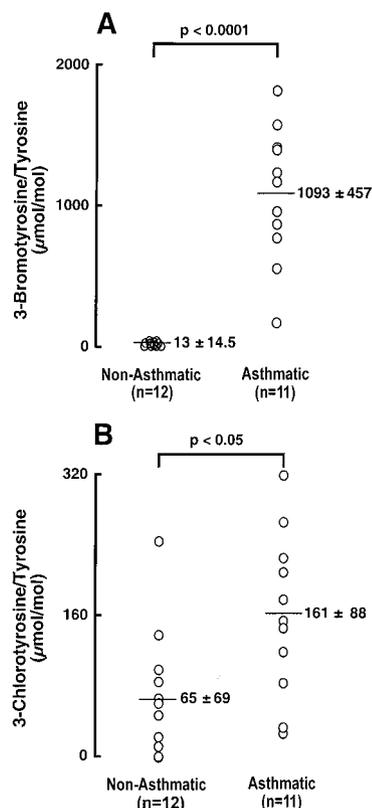


FIGURE 3. Stable isotope GC-MS analyses of BrY and ClY in proteins recovered in endotracheal/bronchial aspirates from severe asthmatic and nonasthmatic subjects. The content of BrY (A) and ClY (B) in proteins recovered from endotracheal/bronchial aspirates from severe asthmatics and nonasthmatic subjects was determined by stable isotope dilution GC-MS as described in *Materials and Methods*. Data points represent the mean of duplicate determinations of samples from each individual. Numbers in parentheses represent the number of subjects in each group. Mean values \pm SD for each group are shown. Substantially higher levels of BrY ($p < 0.0001$) and ClY ($p < 0.05$) were observed for asthmatic vs nonasthmatic subjects.

neutrophils toward oxidation of proteins, revealed a 30-fold difference in asthmatics compared with nonasthmatics (ratio of 6.8 vs 0.2 for asthmatic vs nonasthmatic, respectively).

Human eosinophils nitrate tyrosine in the presence of physiological levels of halides and NO_2^-

We recently demonstrated that isolated EPO effectively uses NO_2^- at concentrations comparable to those observed in inflammatory tissues and fluids ($\leq 50 \mu\text{M}$; Ref. 38 and references therein) in cell-free systems to generate a RNS capable of promoting tyrosine nitration (44). Based upon these studies, we hypothesized that eosinophils might use EPO to contribute to protein oxidation through nitration in asthma. However, the ability of human eosinophils to generate NO-derived oxidants has not yet been demonstrated. To determine whether eosinophils can generate NO-derived oxidants, we isolated peripheral cells from normal healthy donors and incubated them in medium containing L-tyrosine, plasma levels of halides (Medium A, see *Materials and Methods*), and the agonist PMA. Analysis of medium revealed that no significant NO_2Y was formed (Fig. 4A). Moreover, we were also unable to detect any endogenous NO production or significant (i.e., $>1 \mu\text{M}$) $\text{NO}_2^-/\text{NO}_3^-$ accumulation by human eosinophils freshly isolated from peripheral blood, with or without phorbol ester activation, under the conditions and time course used (data not shown). In contrast, eosinophils activated in Medium A supplemented with pathophysiologically relevant levels of NO_2^- ($50 \mu\text{M}$) readily produced NO_2Y (Fig. 4A). The time course for NO_2Y formation paralleled the time course for $\text{O}_2^{\cdot-}$ production during a respiratory burst (Fig. 4B). Finally, in separate studies, eosinophils were activated with an alternative agonist, *N*-formyl-methionyl-leucyl-phenylalanine (100 nM). Cell-dependent NO_2Y formation again demonstrated an absolute requirement for exogenous NO_2^- , although NO_2Y levels produced were ~ 8 -fold less than that observed with eosinophils stimulated with PMA (data not shown).

The absolute requirement of NO_2^- for NO_2Y formation suggested that, under the conditions used, eosinophils were generating RNS via the EPO- H_2O_2 - NO_2^- system. To further explore the reaction mechanism for eosinophil-mediated aromatic nitration reactions, isolated human eosinophils were incubated in medium containing plasma levels of halides (100 mM Cl^- , 100 μM Br^-), levels of NO_2^- observed in epithelial lining fluid of severe asthmatics (50

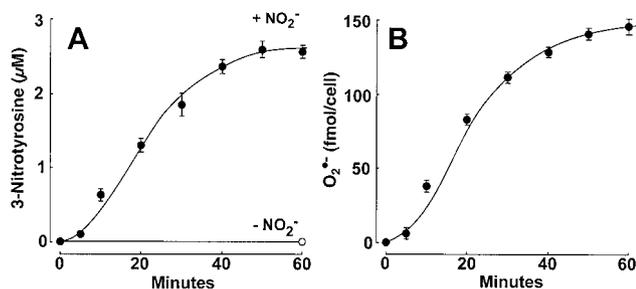
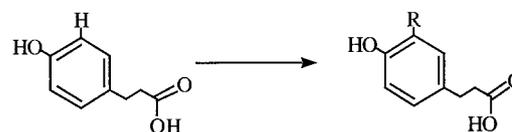


FIGURE 4. Tyrosine nitration and superoxide production by activated eosinophils. *A*, Isolated human eosinophils were activated with PMA (200 nM) in medium containing tyrosine (100 μM) and the presence or absence of NaNO_2 (50 μM) at 37°C for the indicated time intervals. Production of nitrotyrosine was then determined by reversed phase HPLC as described in *Materials and Methods*. Data represent the mean \pm SD of triplicate determinations for a characteristic experiment performed at least three times. *B*, Superoxide anion production was measured following eosinophil activation under similar conditions except in the absence of tyrosine and NaNO_2 in medium, as described in *Materials and Methods*. Data represent the mean \pm SD of triplicate determinations for a characteristic experiment performed at least three times.



R	Oxidation Product	Abbreviation
Cl	3-(3-chloro-4-hydroxyphenyl)propanoic acid	Cl-HPA
Br	3-(3-bromo-4-hydroxyphenyl)propanoic acid	Br-HPA
NO_2	3-(4-hydroxy-3-nitrophenyl)propanoic acid	NO_2 -HPA

FIGURE 5. Use of HPA as a chemical trap to monitor aromatic halogenation and nitration by eosinophils. The deaminated analogue of tyrosine, HPA, was used as a chemical trap to quantify the extent of aromatic nitration and halogenation promoted by activated eosinophils. The structures of the oxidation products monitored are shown.

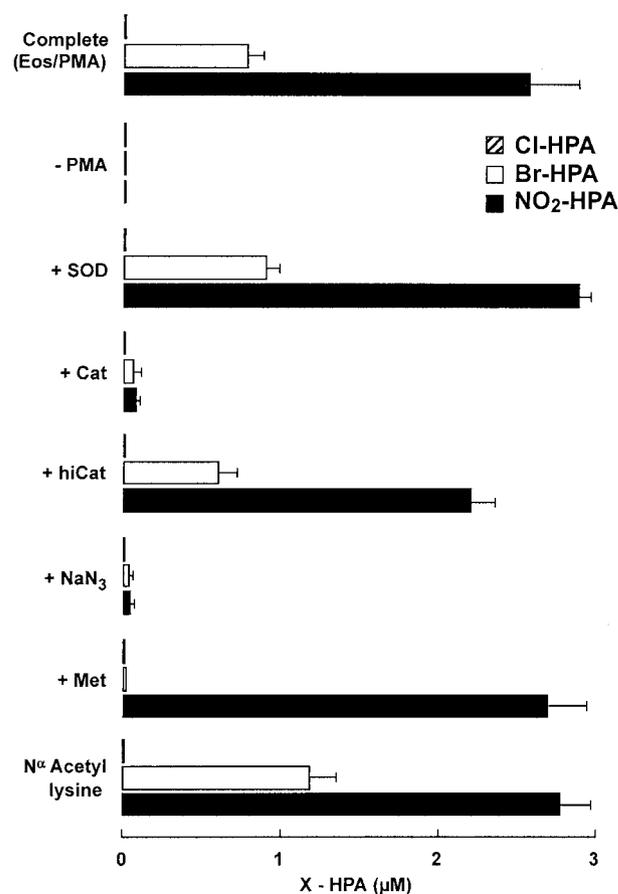


FIGURE 6. Eosinophil-dependent nitration and halogenation of phenolic targets. Human eosinophils (1×10^6 cells/ml) were incubated at 37°C in magnesium-, calcium-, and phenol red-free HBSS supplemented with 100 μM DTPA, 100 μM HPA, 50 μM NaNO_2 , and 100 μM NaBr . Cells were activated by the addition of phorbol ester (200 nM PMA) and maintained in suspension by intermittent inversion for 1 h (Complete System). Additions or deletions to the Complete System were as indicated. Cells were removed by centrifugation, and the capacity of eosinophils to promote aromatic nitration, bromination, and chlorination reactions were determined by quantifying NO_2 -HPA, Br-HPA, and Cl-HPA content in medium as described in *Materials and Methods*. Final concentrations of additions to the Complete System were 10 $\mu\text{g/ml}$ SOD (+SOD), 300 nM catalase (+Cat), 300 nM hiCat (+hiCat), 1 mM azide (+ NaN_3), 1 mM methionine (+Met), and 1 mM N^α -acetyl lysine. Data represent the mean \pm SD of triplicate determinations for a characteristic experiment performed at least three times.

μM), and the deaminated analog of tyrosine, 3-(4-hydroxyphenyl) propanoic acid (HPA, $100 \mu\text{M}$). Use of this low molecular mass surrogate for a protein-bound tyrosine residue permitted more facile quantification of the relative capacity of eosinophils to promote aromatic nitration and halogenation reactions of phenolic targets (Fig. 5).

The capacity of eosinophils to promote aromatic nitration, bromination, or chlorination reactions was assessed by quantifying production of NO_2 -HPA, Br-HPA, and Cl-HPA, respectively, as described in *Materials and Methods*. Following activation by PMA (Complete System, Fig. 6) eosinophils preferentially nitrated HPA, generating ~ 3 -fold more NO_2 -HPA than Br-HPA, and no detectable Cl-HPA (Fig. 6).

Eosinophil-mediated nitration required cell activation and was inhibited by either the presence of the hydrogen peroxide scavenger catalase (but not hiCat) or peroxidase inhibitors (e.g., NaN_3). In contrast, nitration reactions were not blocked by the presence of $\text{O}_2^{\cdot-}$ scavengers (SOD) or scavengers of hypohalous acids (Fig. 6). Similar reaction requirements were observed for eosinophil-mediated bromination reactions (i.e., sensitivity to H_2O_2 scavengers and peroxidase inhibitors) (Fig. 6). One distinguishing feature between eosinophil-mediated nitration and bromination reactions is the inability of the HOBr scavenger methionine to affect nitration by the cells. The lack of inhibition in cell-dependent bromination observed in the presence of the primary amine-containing species, N^α -acetyl lysine, is consistent with our prior observation that N -bromoamines serve as excellent mediators of aromatic bromination reactions (25).

Eosinophils are more efficient than neutrophils at promoting aromatic nitration reactions

In the next series of experiments we compared the ability of purified human eosinophils and neutrophils to nitrate, chlorinate, and brominate the tyrosine analog HPA in the presence of plasma levels of halides over the (patho)physiologically relevant range of NO_2^- (0 – $50 \mu\text{M}$). Eosinophils generated significantly more NO_2 -HPA (>5 -fold) than an equivalent number of neutrophils at all concentrations of NO_2^- examined (Fig. 7). At levels of NO_2^- observed in epithelial lining fluid from normal individuals (i.e., $<10 \mu\text{M} \text{NO}_2^-$), eosinophils were more effective at oxidation of phenolic groups through bromination. As NO_2^- levels became el-

evated into the pathophysiological range ($>10 \mu\text{M}$), nitration of targets predominated (Fig. 7A). In stark contrast to neutrophils, no significant oxidation of HPA through chlorination was observed under all conditions examined (Fig. 7). Finally, neutrophils were ineffective at oxidizing the tyrosine analog through bromination under all conditions examined (Fig. 7).

Eosinophils generate NO-derived oxidants by at least two mechanistically distinct pathways

One striking feature of the results thus far described was that we could find no evidence that isolated eosinophils generate nitrating intermediates through formation of ONOO^- (Equation 2). Based upon the inability to detect NO , NO_2^- , or NO_3^- accumulation in cell medium (see above), this likely reflects the limited capacity of freshly isolated peripheral blood eosinophils from healthy (nonallergic) donors to generate NO , particularly within the brief time period of the respiratory burst (~ 1 h, Fig. 4B). However, enhanced levels of NO in expired breath of asthmatics is well documented, and expression of NO synthase isoforms in the airways is likewise established (26–28). Therefore, we performed a series of experiments in which eosinophils were activated in the presence of an exogenous NO -generating system, PAPA NONOate, and plasma levels of Br^- ($100 \mu\text{M}$). These conditions should more closely mimic a physiological mechanism for NO_2^- formation, as well as provide an environment where eosinophil-generated $\text{O}_2^{\cdot-}$ might react with NO before it dismutates into the EPO substrate, H_2O_2 . Although eosinophils activated in the absence of the NO donor failed to mediate nitration reactions, cells stimulated in the presence of a continuous NO source readily formed NO_2 -HPA (Fig. 8). Aromatic nitration by eosinophils required cell activation, consistent with a requirement for reduced oxygen species ($\text{O}_2^{\cdot-}$ and/or H_2O_2) for oxidation. Moreover, nitration was the favored biochemical pathway for oxidative modification at all but the lowest levels of NO flux examined. At the higher fluxes of NO ($>2 \mu\text{M}/\text{min}$) examined, the overall extent of NO_2 -HPA formation diminished (Fig. 8).

Aromatic nitration reactions mediated by eosinophils in the presence of an exogenous NO donor might occur via formation of either ONOO^- (and/or ONOOCO_2^-) or the EPO- H_2O_2 - NO_2^-

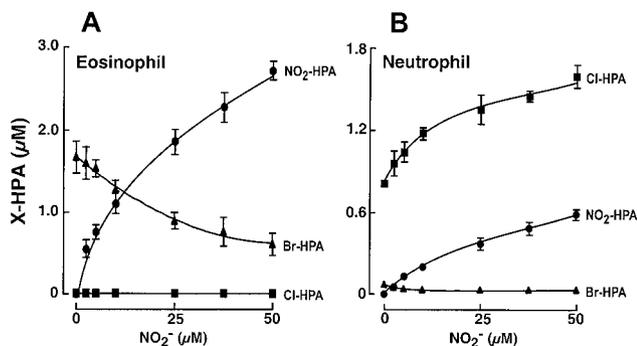


FIGURE 7. Eosinophil and neutrophil modification of HPA at physiologically relevant levels of nitrite. Isolated human eosinophils and (B) neutrophils (1×10^6 cells/ml) were incubated for 1 h at 37°C in magnesium-, calcium-, and phenol-free HBSS supplemented with $100 \mu\text{M}$ HPA, $100 \mu\text{M}$ NaBr, and the indicated concentrations of NaNO_2 . Cells were then pelleted and the content of NO_2 -HPA, Br-HPA, and Cl-HPA generated by eosinophils and neutrophils was then determined as described in *Materials and Methods*. Data represent the mean \pm SD of triplicate determinations for a characteristic experiment performed at least three times.

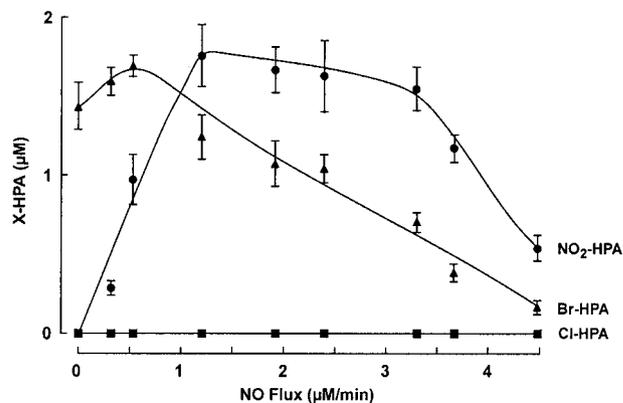


FIGURE 8. Investigation of the potential pathways available to eosinophils for generating NO -derived oxidants. Phorbol ester-stimulated eosinophils (1×10^6 cells) were incubated in calcium-, magnesium-, and phenol red-free HBSS containing $100 \mu\text{M}$ NaBr, $100 \mu\text{M}$ HPA, and the indicated rates of NO release from an exogenous NO source, PAPA NONOate, as described in *Materials and Methods*. The content of NO_2 -HPA, Br-HPA, and Cl-HPA formed was then determined as described in *Materials and Methods*. Data represent the mean \pm SD of triplicate determinations for a characteristic experiment performed at least three times.

system. To gain insights into the pathway(s) used by eosinophils to generate NO-derived oxidants, we examined the effects of EPO inhibitors, H_2O_2 scavengers, and SOD on NO_2 -HPA formation. At low rates of NO flux ($<2 \mu M/min$), NO_2 -HPA formation was inhibited by azide, a heme poison that inhibits EPO catalysis (Fig. 9). Under these same conditions, catalase, but not hiCat, significantly attenuated NO_2 -HPA production, consistent with a role for H_2O_2 in the aromatic nitration reaction. In contrast, at high rates of NO flux ($>2 \mu M/min$), aromatic nitration by activated eosinophils became increasingly less sensitive to inhibition by either azide or catalase. Finally, while addition of SOD to reactions demonstrated a modest increase in NO_2 -HPA formation at low levels of NO flux, inhibition in nitration was observed at the higher rates of NO flux examined (Fig. 9).

Discussion

Enhanced production of NO is widely observed in the exhaled breath of asthmatics (26–28). Because of the ability of NO to generate potent nitrating intermediates, it has been suggested that RNS contribute to inflammatory tissue injury in asthma. However, remarkably few studies have actually directly examined the role of RNS in oxidation of biological targets in human asthma. Evidence thus far reported is primarily limited to studies that rely upon immunohistochemical detection of NO_2Y (32, 33). Because of the inherent uncertainties in the precise cognate epitope recognized by immunohistochemical methods and their nonquantitative nature, we sought to use more direct chemical methods to establish that RNS contribute to oxidative modification of proteins in asthma.

The results of this study directly demonstrate that RNS contribute to oxidative modification of proteins in asthma. They also strongly support a role for eosinophils in generating NO-derived oxidants in severe asthma. Using a specific and sensitive mass spectrometric method, we observe a 10-fold increase in the content

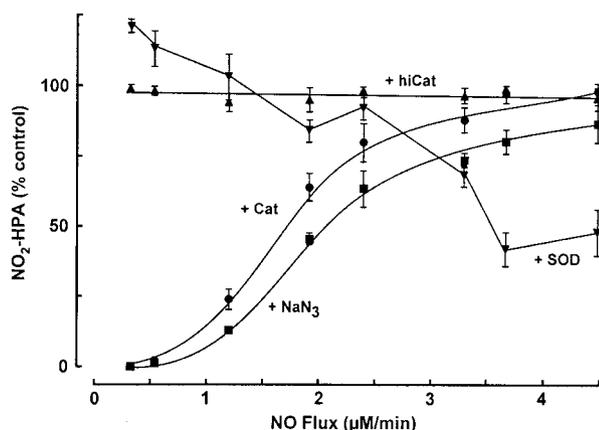


FIGURE 9. Effect of EPO inhibitor, H_2O_2 scavenger, and SOD on eosinophil-mediated aromatic nitration reactions. Phorbol ester-stimulated human eosinophils (1×10^6 cells) were incubated in calcium, magnesium, and phenol-free HBSS containing $100 \mu M$ NaBr, $100 \mu M$ HPA, and the indicated rates of NO release from an exogenous NO source, PAPA NONOate, as in Fig. 8 (control condition). In parallel reactions, cells were also incubated with the indicated additions. Reactions were stopped by removal of cells by centrifugation at $4^\circ C$, and the content of NO_2 -HPA was determined. Data represent the mean \pm SD of triplicate determinations of a representative experiment and are expressed as the percentage of NO_2 -HPA formed in the presence of the indicated additions relative to that observed under control conditions. Similar results were observed in three independent experiments. Concentrations of additives, when present, were as follows: $1 mM$ azide (NaN_3), $300 nM$ catalase (+Cat), $300 nM$ hiCat (+hiCat), $10 \mu g/ml$ SOD.

of NO_2Y in proteins recovered from airways of patients with severe asthma compared with nonasthmatic subjects (Fig. 1). Eosinophil activation and subsequent protein oxidation in the asthmatic airways is also supported by the dramatic increase in BrY content observed in airway proteins recovered from asthmatic subjects admitted to the intensive care unit (Fig. 3). Moreover, immunohistochemical studies implicate eosinophils as a major cellular generator of NO-derived oxidants in severe asthma because epitopes demonstrating NO_2Y -specific immunostaining substantially colocalize with the leukocyte (Fig. 2). Finally, studies with isolated human eosinophils reveal that aromatic nitration reactions are a preferred oxidation pathway mediated by the leukocytes at plasma concentrations of halides and levels of nitrite observed in airway lining fluid of asthmatics (Figs. 6 and 7).

One of the remarkable findings of these studies is that human eosinophils can use distinct chemical mechanisms for generating NO-derived oxidants and that the relative preference of eosinophils for promoting aromatic nitration vs halogenation reactions varies depending upon their environment during activation. Moreover, the environment in which eosinophil activation occurs (e.g., rate of NO production) also influences the relative contribution that chemically distinct oxidation pathways play in contributing to protein nitration. On the basis of these results and recently published studies (24, 25, 44), we have generated the following model (Fig. 10) of pathways available to human eosinophils for oxidizing proteins through formation of reactive halogen and RNS. During eosinophil activation, such as following allergen challenge, the phagocyte undergoes a respiratory burst, generating $O_2^{\cdot-}$ and its dismutation product, H_2O_2 . Concomitantly, the contents of the secretory granules are released, including EPO. EPO ($15 \mu g/10^6$ eosinophils) constitutes $\sim 25\%$ of the total protein mass of specific granule protein, and is thus one of the most abundant proteins in eosinophils (61). When human eosinophils are activated in medium possessing plasma levels of halides and low levels of NO (and its autooxidation product, NO_2^-), eosinophils preferentially use the EPO- H_2O_2 system to generate brominating oxidants (Figs. 6 and 8). Aromatic bromination reactions mediated by the activated cells are inhibited in the presence of the HOBr scavenger methionine, a thioether, but not by large molar excesses of primary amines (Fig. 6). These results are consistent with our prior observations that *N*-mono-bromoamines are preferred brominating intermediates for protein tyrosine residues in cell-free systems at neutral pH (44). The ability of *N*-mono-bromoamines to promote phenolic bromination reactions across the (patho)physiological pH range also likely contributes to the relatively high levels of protein BrY observed in endotracheal/bronchial aspirates recovered from patients with severe asthma (Fig. 3).

Under these same conditions (i.e., low levels of NO flux ($<2 \mu M/min$), plasma levels of halides), the predominant pathway for

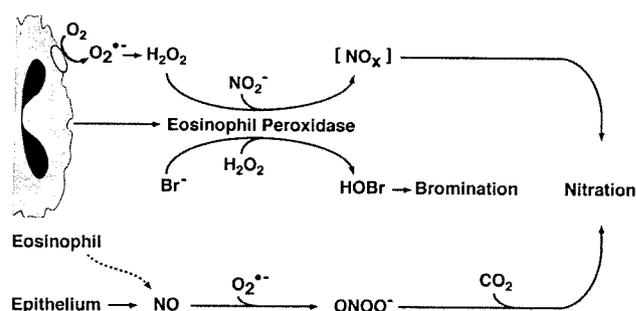


FIGURE 10. Model of potential pathways used by eosinophils for the generation of NO-derived oxidants and reactive halogen species.

generating NO-derived oxidants by eosinophils appears to be via oxidation of NO_2^- by the EPO- H_2O_2 pathway (Fig. 10). The ability of human eosinophils to use this pathway is strongly supported by the demonstration that activated eosinophils (this study) and isolated EPO (44) use physiologically relevant levels of NO_2^- to promote nitration of phenolic targets as a preferred activity. A primary role for EPO- and H_2O_2 -dependent formation of RNS by eosinophils is further supported by 1) the absolute requirement of NO_2^- for aromatic nitration reactions (Figs. 4 and 7); and 2) the ability of EPO inhibitors (NaN_3) and H_2O_2 scavengers (catalase, but not hiCat) to block phenolic nitration (Figs. 6 and 9). The RNS formed by EPO-catalyzed oxidation of NO_2^- has not been identified. Studies with the related hemoprotein MPO suggest that the one electron oxidation product, nitrogen dioxide ($\cdot\text{NO}_2$), is a likely product (38, 40, 62). It should also be noted that isolated human EPO promotes nitration of free and protein-bound tyrosine residues at least 4- to 5-fold more efficiently than human MPO (on an equimolar basis, which corresponds to a 8- to 10-fold difference per heme group) (44).

Another remarkable finding of this study is the overall high yield of tyrosine nitration by eosinophils via the EPO- H_2O_2 - NO_2^- pathway. If one assumes that 2 mol of $\text{O}_2^{\cdot-}$ are required to form 1 mol of H_2O_2 during the respiratory burst, and 2 mol of H_2O_2 are required per mol of NO_2Y formed (one to generate $\cdot\text{NO}_2$ and another to generate tyrosyl radical; Ref. 63), then the overall yield of eosinophil-dependent nitration of tyrosine via the EPO- H_2O_2 - NO_2^- system was $\sim 6.6\%$ under the conditions used in Fig. 4. Finally, although formation of a nitrating and halogenating species through secondary oxidation of NO_2^- by hypohalous acids has been suggested for the MPO-generated product HOCl (64), eosinophil-mediated nitration of HPA in the presence of NO_2^- was not blocked by addition of scavengers of halogenating oxidants (thioethers and amines, Fig. 6). Thus, eosinophil-generated HOBr does not appear to play a significant role in subsequent formation of NO-derived oxidants by these cells.

Isolated peripheral blood eosinophils from normal healthy donors failed to generate detectable levels of NO, NO_2^- , or NO_3^- during the brief incubation periods used in this study. Consistent with these results, eosinophil activation in the absence of an exogenous source of either NO_2^- or NO failed to nitrate phenolic targets (Figs. 4, 7, and 8). However, eosinophil activation in vivo occurs in an environment replete in NO, whether generated by cytokine-stimulated eosinophils or adjacent cells. As noted above, in the presence of an exogenous NO donor, eosinophils readily promoted aromatic nitration reactions (Figs. 8 and 9). At high rates of NO flux ($>2 \mu\text{M}/\text{min}$), eosinophils appear to use a pathway for generating RNS that is chemically distinct from the EPO- H_2O_2 - NO_2^- system because EPO inhibitors and H_2O_2 scavengers do not significantly inhibit phenolic nitration reactions under these conditions, yet addition of SOD does (Fig. 9). These results are consistent with interaction of NO with cell-generated $\text{O}_2^{\cdot-}$ forming ONOO $^-$ and ONOOCO $_2^-$ (Equation 2; Fig. 10). The relative contribution of the ONOO $^-$ /ONOOCO $_2^-$ vs the EPO- H_2O_2 - NO_2^- system for eosinophil-dependent generation of NO-derived oxidants during asthma is unknown. However, the high levels of BrY observed in BAL proteins from severe asthmatics suggest that the EPO pathway may play a significant role in protein oxidation and, therefore, NO_2Y formation. At the levels of NO_2^- typically observed in epithelial lining fluid during severe asthma ($>10 \mu\text{M}$), nitration of phenolic targets by the EPO system of eosinophils is preferred (Fig. 7). Finally, it should be noted that a wide range of steady-state NO levels are observed in vivo (20 nM-2 μM ; Ref. 30), and NO-dependent protein nitration was mediated by activated eosinophils over this range (Fig. 8).

One interesting finding in this study is that the overall yields of nitration and bromination decline at higher fluxes ($>2 \mu\text{M}/\text{min}$) of NO (Fig. 8). Similar bell-shaped curves for the overall extent of protein and lipid oxidation mediated by leukocytes (e.g., neutrophils and monocytes) activated in the presence of varying levels of NO recently have been reported (39, 41). One possible explanation is that NO modulates the extent of oxidation by interacting with critical heme proteins involved in $\text{O}_2^{\cdot-}$ formation (e.g., NADPH oxidase) or RNS formation (e.g., EPO). Recent studies demonstrate that NO modulates the catalytic activity of the related peroxidase MPO (65). At low levels, NO serves to enhance catalytic rates by accelerating the rate limiting step in the peroxidase cycle, reduction of Compound II (65, 66). At higher levels of NO, MPO activity is inhibited by formation of a ferric-nitrosyl complex (65). Similar behavior may occur during NO interactions with EPO. Another potential mechanism accounting for the inhibition in the extent of oxidation at higher fluxes of NO is that it may partially act as an antioxidant under these conditions by scavenging reactive intermediates through radical-radical interactions (67). Alternatively, decreased formation of nitrotyrosine at high fluxes of NO may be due to lower overall yields with ONOO $^-$ /ONOOCO $_2^-$ compared with the RNS formed by the EPO- H_2O_2 - NO_2^- system.

Recent studies by Gaston and colleagues demonstrate that airway vapor condensates from severe asthmatic subjects are acidic (68). This has led to the suggestion that elevated levels of NO observed in asthmatic subjects may arise in part from protonation of NO_2^- (68, 69). Although the pH of airway lining fluids in the subjects examined in this study were not determined, it is likely that they were acidic as well. Therefore, one might speculate that some of the NO_2Y formed on BAL proteins during asthma arose from nonenzymatic formation of RNS. However, several lines of evidence suggest that eosinophils are a major source of NO-derived oxidants in vivo and that EPO plays an active role in NO_2Y formation during asthma. Perhaps the strongest evidence is the intense focal staining observed in bronchial biopsies probed with Abs specific for NO_2Y where the immunostaining predominantly colocalized with eosinophils (Fig. 2). Moreover, using mass spectrometry, we recently demonstrated that significant levels of bromination (25) and nitration (44) of target proteins incubated in medium containing plasma levels of halides and pathophysiologically relevant levels of NO_2^- does not occur through nonenzymatic processes to any significant degree over the pH range observed in normal and asthmatic human airways (pH 5.5-7.5). Moreover, analysis of halogenated tyrosine adducts in protein present in lavage fluids from this study revealed a much higher proportion of BrY than ClY in severe asthmatics vs controls subjects (Fig. 3), despite the 1000-fold higher concentration of Cl $^-$ observed in plasma. Given that eosinophils selectively brominate while neutrophils chlorinate proteins at plasma levels of halides (Fig. 7 and Ref. 24), these in vivo data strongly support the notion that eosinophils are likely a major leukocyte responsible for promoting oxidative modification of proteins in asthmatic patients. The calculation of the BrY/ClY ratio may thus represent an objective and quantifiable index to estimate the relative contributions of eosinophils vs neutrophils in oxidative modification of proteins in tissues. Finally, these studies with isolated human eosinophils clearly demonstrate that the leukocytes readily form NO-derived oxidants under physiologically relevant conditions.

Measurement of NO_2Y is now widely used as a marker for protein oxidation by RNS. A multitude of techniques for quantification of NO_2Y in biological tissues and fluids are used, including immunoassay and a variety of HPLC and mass spectrometry-based methods. The validity of many of these methods has been

questioned because of the ease with which artificial phenolic nitration occurs in the presence of nitrite and acid pH. Only mass spectrometry combined with isotope-labeling techniques permits simultaneous monitoring of authentic oxidized amino acids (e.g., NO₂Y) and intrapreparative formation of the analyte during sample handling (24, 41, 70, 71). The stable isotope dilution mass spectrometric methods used in this study excluded any significant contribution of ex vivo nitration (as well as bromination and chlorination) of airway proteins. It is interesting to note that levels of NO₂Y observed in proteins recovered from airways of nonasthmatic subjects in this study are extremely low, similar to those recently observed on induced sputum of normal subjects (72) and in other tissues and fluids examined using mass spectrometry-based methods that permit development of sample preparation methods that minimize artifactual nitration (70, 71).

It should be noted that the biological consequences of protein oxidation via nitration or bromination during asthma are not known. However, it should also be appreciated that the stable covalent adducts of tyrosine monitored represent only a fraction of the total modifications incurred during exposure of proteins to reactive nitrogen or halogenating species. Other nucleophilic targets on proteins including thiol (cysteine), thioether (methionine), imidazole (histidine), and indole (tryptophan) groups are all targets for oxidation by either brominating or nitrating agents. The chemical reactivity of EPO-generated oxidants undoubtedly plays a significant role in the cytotoxic properties of these agents on pathogens and eukaryotic cells. Thus it is reasonable to speculate that oxidative modification of critical biological targets in asthmatic airways may contribute to the pathophysiological sequelae of the disease. Particularly given the known toxicity of HOBr and the EPO-H₂O₂-Br⁻ system to eukaryotic cells in culture (73, 74), and their capacity to elicit many of the pathophysiological features of asthma, such as epithelial cell damage and sloughing, airway hyperreactivity, bronchoconstriction, β -adrenergic dysfunction, mucus hyper secretion, microvascular leak, and edema (75–80).

Collectively, these data suggest that eosinophils contribute to oxidative protein modification in lung and airways of asthmatics. They also further define chemical pathways available to eosinophils for contributing to inflammatory injury in vivo and reveal that these unique effector cells in the host response can use a variety of distinct chemical pathways to promote oxidative modification of targets. Knowledge of the precise chemical pathways used by eosinophils is critical to the development of targeted interventions designed to retard or interrupt these processes. One such target that is suggested by these studies is EPO. Future work is warranted to assess the potential clinical impact of EPO inhibition in human asthma.

Acknowledgments

Mass spectrometry experiments were performed at the Mass Spectrometry Facility of the Cleveland Clinic Foundation.

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