EOSINOPHIL PEROXIDASE OXIDATION OF THIOCYANATE:
CHARACTERIZATION OF MAJOR REACTION PRODUCTS AND A
POTENTIAL SULFHYDRYL-TARGETED CYTOTOXICITY SYSTEM

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SUMMARY

Although the pseudohalide thiocyanate (SCN⁻) is the preferred substrate for eosinophil peroxidase (EPO) in fluids of physiologic halide composition, the product(s) of this reaction have not been directly identified and mechanisms underlying their cytotoxic potential are poorly characterized. We used nuclear magnetic resonance spectroscopy (NMR), electrospray ionization mass spectrometry (ESI-MS), and quantitative chemical analysis to identify the principal reaction products of both the EPO/SCN⁻/H₂O₂ system and activated eosinophils as roughly equimolar amounts of OSCN⁻ (hypothiocyanite) and OCN⁻ (cyanate). Red blood cells (RBCs) exposed to increasing concentrations of OSCN⁻/OCN⁻ are first depleted of glutathione, after which glutathione-S-transferase (GST) and glyceraldehyde-3-phosphate-dehydrogenase, then ATPases undergo sulfhydryl (SH) reductant-reversible inactivation prior to lysing. OSCN⁻/OCN⁻ inactivates RBC membrane ATPases 10-1000 times more potently than do HOCl, HOBr, and H₂O₂. Exposure of GST to [¹⁴C] OSCN⁻/OCN⁻ causes SH-reductant-reversible disulfide bonding and covalent isotope labeling. We propose that EPO/SCN⁻/H₂O₂ reaction products comprise a potential SH-targeted cytotoxic system that functions in striking contrast to HOCl, the highly but relatively indiscriminantly reactive product of the neutrophil myeloperoxidase system.
ABBREVIATIONS

**EO**: eosinophil; **EPO**: eosinophil peroxidase; **ESI-MS**: electrospray ionization mass spectometry; 
**GAPDH**: glyceraldehyde-3-phosphate dehydrogenase; **GST**: glutathione-S-transferase; **LPO**: lactoperoxidase; **MPO**: myeloperoxidase; **MS/MS**: tandem mass spectometry; **NMR**: nuclear magnetic resonance; **PBS**: phosphate-buffered saline, pH 7.4; **PMA**: phorbol myristate acetate; 
**RBC**: red blood cell; **SDS/PAGE**: sodium dodecyl sulfate/polyacrylamide gel electrophoresis; 
**SH**: sulfhydryl; **TNB**: 5-thio-2-nitrobenzoic acid
INTRODUCTION

Human phagocytes utilize respiratory burst-derived $\text{H}_2\text{O}_2$ in combination with a peroxidase and halides to generate reactive oxidants that can kill bacterial, fungal, metazoan, and viral pathogens but also damage host tissue. The overall reaction is: $\text{H}_2\text{O}_2 + \text{X}^- + \text{H}^+ \rightarrow \text{HOX} + \text{H}_2\text{O}$, where $\text{X}^- = \text{Cl}^-, \text{Br}^-, \text{SCN}^-, \text{or I}^-$ and HOX is the corresponding hypohalous acid. Two distinct human phagocytic peroxidases have been identified: neutrophil myeloperoxidase (MPO) and a related [70% amino acid homology (1,2)] eosinophil peroxidase [EPO (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7)]. Neutrophil MPO predominantly catalyzes the two-electron oxidation of the most abundant halide, Cl$, to generate the potent bleaching oxidant hypochlorous acid (HOCl), a highly but indiscriminately reactive substance with potent cytocidal capacity. This powerful "bleaching" oxidant reacts rapidly with a wide variety of cell membrane components, disrupts membrane integrity and lyses cells (3-5). In view of these characteristics, HOCl seems well suited for fulfilling the primary function of the neutrophil: to kill relatively small internalized microbes within the confines of a phagocytic vacuole.

In contrast to neutrophils, eosinophils (EOs) mediate the extracellular destruction of large metazoan pathogens such as helminthic parasites (6). Eosinophils contain large quantities [15 $\mu$g/10$^6$ eosinophils, 40% by weight of total specific granule protein (7,8)] of EPO, a 77,000 dalton, two-subunit enzyme which, though related to MPO, is both structurally (1,2,8) and functionally (9-12) distinct from MPO. EPO plays a critical role in EO killing of parasites under physiologic conditions because EPO inhibitors nearly completely block the killing of antibody- and complement-opsonized schistosomules by intact eosinophils in serum conditions (13).

The physiologic substrate for eosinophil EPO, though clearly different from that of MPO, is less certain. EPO oxidizes Cl$^-$ poorly, if at all (9-12). In physiologic fluids such as serum and extracellular fluid the concentrations of halides are 100 mM Cl$^-$, 20-100 $\mu$M Br$^-$, 20-120 $\mu$M SCN$^-$, and <1 $\mu$M I$^-$ (14-16). Considering other potential substrates, it has been shown the EPO/I$^-$...
/H₂O₂ system has pronounced toxicity for bacteria (9), parasites (17,18), and mammalian cells (10,19), but only at I⁻ concentrations far above physiologic. Alternatively, in the presence of physiologically relevant concentrations of Cl⁻ and Br⁻ (i.e., 100 mM and 20-100 µM, respectively) EPO preferentially oxidizes Br⁻ to form another potent bleaching oxidant, HOBr, suggesting that Br⁻ might be the predominant natural substrate (20,21,75,76). However, other work has shown (9,10,22,23) that the pseudohalide thiocyanate (SCN⁻) can also be a substrate for the EPO system. We found (23) that, in fluids of physiologic halide composition (i.e., 100 mM Cl⁻, 20-100 µM Br⁻, 10-100 µM SCN⁻, and 1 µM I⁻) and in serum, SCN⁻ is the virtually exclusive substrate for EPO oxidation, both by purified EPO and by activated eosinophils. Nitrite (NO₂⁻) can also serve as a substrate for EPO, producing a bactericidal product (77) that nitrates protein tyrosine residues (78); but SCN⁻ is preferentially oxidized over NO₂⁻ as well (78). Based upon these data, we propose that, unexpectedly, SCN⁻ is the primary substrate oxidized by EPO and H₂O₂ under physiologic circumstances.

The product(s) of this potentially important reaction have, however, not yet been directly identified. Previous work (22,25,26) on the closely related lactoperoxidase/SCN⁻/H₂O₂ system has shown that it generates a relatively weak, predominantly sulfhydryl (SH)-reactive oxidant that is both bacteriostatic and bactericidal. By analogy with the other halide/hypohalous systems, it was proposed that this oxidant might be HOSCN, or hypothiocyanous acid. The product(s) of the EPO/SCN/H₂O₂ system react(s) with the sulfhydryl dye 5-thio-2-nitrobenzoic acid (TNB) (22-26) and is profoundly less toxic (on a molar basis) for mammalian cells than is HOBr or HOCl (23,27), a profile similar to the product(s) of the lactoperoxidase/SCN⁻/H₂O₂ system. However, even in the better-characterized lactoperoxidase/SCN⁻/H₂O₂ system, based upon nuclear magnetic and electron spin resonance studies, at least four different compounds have been proposed to be the major reaction products: HOSCN (28,29); CN⁻ (29); NCS-O-SCN (28); and 'OSC•N⁻ (30). We now use nuclear magnetic resonance (NMR) and electrospray ionization mass spectometry (ESI-MS) to identify the major stable reaction products of the EPO/SCN/H₂O₂ system and demonstrate its capacity to inflict sulfhydryl reactivity–based toxicity. These findings suggest that
the eosinophil EPO system employs a radically different biologic strategy to kill extracellular pathogens than the neutrophil MPO system does to kill intracellular pathogens.
Experimental Procedures

**Materials.** Human EPO was kindly provided by Dr. Gerald J. Gleich (Mayo Clinic and Research Foundation, Rochester, MN). EPO was isolated from granule extracts of purified eosinophil suspensions obtained from patients with hypereosinophilic syndrome as previously described (31). Granule extracts were then chromatographed on a Sephadex G-50 column equilibrated with 0.25 M acetate buffer (pH 4.3, 0.15 M NaCl). The fractions eluting with the void volume were then collected and purified to an OD\(_{415/280}\) ratio of >0.9 by chromatography on carboxymethyl Sepharose as described by Carlson et al. (8). SDS-polyacrylamide gel electrophoresis was used to confirm homogeneity of the EPO prep. Two discrete bands (molecular mass of ~78 and ~14 KD) that correspond to the heavy and light chains of EPO, with no contaminating bands, were present. EPO activity was assayed with guaiacol oxidation and converted to international units (the amount of the enzyme that oxidizes 1 µmol of electron donor/min at 25° C (32). The specific activity of the EPO utilized in the course of these experiments was 133-250 U/mg protein. Preparations were stored at -70° until needed for use and were then maintained at 4° wrapped in foil. [\(^{13}\)C]-labeled (99-atom %) potassium thiocyanate was obtained from Cambridge Isotope Laboratories (Andover, MA). Unlabelled potassium thiocyanate was from Fisher Scientific (Pittsburgh, PA). \([^{14}\text{C}]\text{SCN}\) as the potassium salt, specific activity 55.1 mCi/mmol, was from Amersham (Arlington Heights, IL). Potassium cyanate was from Baker (Phillipsburg, NJ). Percoll was from Pharmacia Fine chemicals (Piscataway, NJ). CD-16 magnetic microbeads were obtained from Miltenyi Biotech Inc (Sunnyvale, CA). PMA was from Consolidated Midland Corporation (Brewster, NY). Hank’s Buffered Salt solution (HBSS) was obtained from Gibco Laboratories (Grand Island, NY). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

**Temperature- and pH-dependence of EPO/SCN/H\(_2\)O\(_2\) system oxidants.** Oxidants were quantified by titration of thionitrobenzoic acid (TNB) as previously described (22). TNB was generated from 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) by addition of 2 µl \(\beta\)-
mercaptoethanol to 50 ml of a 1 mM DTNB solution. Freshly prepared TNB is calibrated at $A_{412}$ and combined with EPO reaction mixture to a total volume of 1 ml to determine HOSCN-/OSCN-concentration using a molar extinction coefficient of 26,000 M$^{-1}$cm$^{-1}$. Independent studies with reagent OCN$^-$ confirmed that this assay does not detect OCN$^-$ (not shown). EPO-catalyzed oxidation of SCN$^-$ was performed using conditions similar to those described by Modi et al. using the lactoperoxidase system. KSCN (2 mM) and EPO (0.4 µM) were added to in 0.1 M sodium phosphate buffer at pH 6.0 or 7.4 (total volume 1 ml) and **five consecutive 250 µM increments of H$_2$O$_2$ were added at one minute intervals at room temperature (25°C), vortexing between additions.** One minute after the final bolus addition, 20 µl of catalase (2 mg/ml) was added to consume excess unreacted H$_2$O$_2$. Specimens were incubated then at 25° or 4° and after 0, 30, 90, 120, and 240 min, 100 µl aliquots were removed for assay of oxidants by TNB titration.

**NMR analysis of EPO/[¹³C]SCN/H$_2$O$_2$ system reaction products and [¹³C] urea.** The [¹³C] SCN$^-$ spectra were collected either on a Bruker AMX-600 MHz apparatus using an 8 mm broad-band probe or on Varian Inova-600 using 5 mm broad-band probe. Spectra were obtained of EPO/[¹³C] SCN$^-$/H$_2$O$_2$ reaction mixture in 10% D$_2$O 90% 0.1M sodium phosphate buffer at either pH 6.0 or 7.4. Reactions were mixed as described above to 2 ml total volume at appropriate pH at room temperature. One minute after final H$_2$O$_2$ bolus addition, 50 µl of catalase (2 mg/ml) was added to reaction tube and reaction mixtures were transferred to ice. Samples were placed in an 8 inch, 8 mm thin-walled NMR sample tube (Wilmad Glass, Buena NJ). 1D spectra were collected using the following parameters, sw= 15,009, number of points = 32,000, acquisition time = 1.066s, recycle delay of 2s, number of scans either 1500 or 2000. The data were processed using gaussian function 0.099. Chemical shifts were referenced relative to external (CH$_3$)$_4$Si. For determination of cyanate spectrum, a 1 M solution of [¹³C]-labeled urea was prepared in PBS buffer (pH 7.4) supplemented with 10% D$_2$O and incubated for 40 minutes in a water bath at 85°C to promote equilibration between urea and cyanate. Assay of OCN$^-$ by chemical analytic assay (see below) in this preparation confirmed a cyanate concentration of 5 mM (not shown). NMR spectra were then obtained as described above.
Electrospray ionization mass spectrometry (ESI-MS) and collision-induced dissociation analysis of the EPO/SCN⁻/H₂O₂ system reaction products. Negative ion ESI-MS was carried out on a triple stage quadrupole mass spectrometer (Perkin Elmer SCIEX API III, Foster City, CA, USA). The instrument was tuned and calibrated for negative ion operation using polypropylene glycol with the Sciex IonSpray® source. EPO (0.4 µM) and either [13C] SCN⁻ or [12C] SCN⁻ potassium thiocyanate (2 mM) were combined in 10 mM ammonium acetate buffer pH 7.4. Five 200 µM boluses of H₂O₂ were added at room temperature at one minute intervals with mixing and 40 µg/ml of catalase was added to destroy excess H₂O₂. The samples were then placed in Microcon concentrators (Amicon, MA, USA) with a molecular weight cutoff of 10 kDa, centrifuged for 30 minutes (13,000 x g at 4°C) to remove protein, and kept cold until ESI-MS analysis in the negative ion mode. Alternatively, for some experiments samples were filtered through a 0.22 µM exclusion Acrodisc to remove particles and immediately analyzed. Samples were infused into the IonSpray® source at a flow rate of 5-10 µL/min using a syringe pump (Harvard Apparatus Model 22, South Natick, MA, USA). The various instrumental parameters involved in efficient nebulization and charging of droplets were optimized to the following values: spray needle voltage (ISV, −3500V); interface plate (IN, −650V); orifice skimmer (OR, −50 to −100V); Q0 rod offset voltage (RO, −30V); gas curtain interface (1.2 L/min of N₂ at 60°C); nebulizer gas (air at 1.5 L/min). Data were acquired in the range of 10 to 100 m/z at a step size of 0.2 AMU and 5 to 10 msec dwell time; 10 to 30 scans were summed over time. Collision-induced dissociation tandem mass spectrometry (MS/MS) studies were performed on a Micromass Quatro II triple quadrupole mass spectrometer (Altrincham, U.K.). Collision-induced mass spectra were determined in the negative ion mode by direct infusion of reaction products (formed by the EPO/SCN⁻/H₂O₂ system as described for the ESI/MS studies above) at a flow rate of 10 µl/min (Harvard Apparatus pump) and analyzed with a cone potential of 20 eV, collision energy of 30 eV, collision gas (Ar) cell at 1.7 x 10⁻³ mbar, source temperature 70°C, capillary 3500 V, high voltage lens 690V, skimmer offset of 5 V.
Analytic quantitation of cyanate. OCN⁻ was assayed using the method of Guilloton and Karst (33). Briefly, 250 ml of a freshly prepared 10 mM solution of 2-aminobenzoic acid (anthranilic acid) prepared in 50 mM sodium phosphate buffer at pH 4.4 was incubated with 250 µl of the EPO system reaction mixture for 10 minutes at 40° and then added to 500 µl 10N HCl. The mixture was then boiled for one minute, allowed to cool to room temperature and assayed spectrophotometrically at 310 nm with a molar extinction coefficient of 3.56 mM⁻¹cm⁻¹. A standard curve using reagent KOCN was used to determine specific concentration. This assay does not detect HOSCN/OSCN⁻ because EPO system reaction mixtures contained the same amount of cyanate before and immediately after selective titration of HOSCN/OSCN⁻ by addition of dithiothreitol (not shown).

Production of oxidants and cyanate by the EPO/SCN⁻/H₂O₂ system: time course and peroxidase dependence. To determine the relative time courses of HOSCN and cyanate generation (Figure 5), H₂O₂ was added to a reaction mixture containing PBS with 1 mM KSCN, pH 7.4, and 0.4 µM EPO by continuous infusion at 50 µM/min for 20 min to simulate the continuous generation of H₂O₂ by the respiratory burst of an activated eosinophil. An aliquot was removed from the reaction mixture prior to and at various time intervals after initiating addition of H₂O₂, supplemented with 40 µg/ml catalase to consume unreacted H₂O₂, and placed on ice until subsequent assay of oxidants and cyanate as described above. To establish the peroxidase-dependence of oxidant and cyanate generation by the EPO/SCN⁻/H₂O₂ system (Figure 7), the effect of either omitting EPO or adding the EPO inhibitor azide (1mM) was assayed in a system comprised of PBS (pH 7.4), 0.4 µM EPO, and 1 mM KSCN to which five consecutive increments of 100 µM H₂O₂ were added at one minute intervals, and the reaction was terminated by adding 100 µg/ml catalase and placing the specimen on ice prior to assaying oxidants and cyanate.

Purification of peripheral blood eosinophils. The anti-CD-16 immunomagnetic bead cell sorting system (MACS, Miltenyi Biotec Inc.) was used as described by Ide et al. (34). Minor modifications are delineated here. 60 ml of citrated blood from eosinophilic donors (n=3)
undergoing interleukin-2 immunotherapy were mixed with 30 ml of 6% Hetastarch in a 0.9% Sodium Chloride solution (Abbott Labs, IL). Erythrocytes were allowed to sediment for 45 minutes at room temperature and the leukocyte-rich fraction was collected. This fraction was diluted with an equal volume of phosphate buffered saline (PBS) [137 mM NaCl, 3 mM KCl, 4.2 mM Sodium phosphate and 1.5 mM potassium phosphate, pH 7.4] supplemented with 2% fetal bovine serum, layered atop a half-volume of isotonic Percoll (density 1.082 g/ml) in 50 ml conical tubes, and centrifuged for 30 minutes, 1000 x g at 4°C. The supernatant and mononuclear cells at the interface were carefully aspirated and the inside wall of the tube wiped with sterile cotton tip applicators to remove residual mononuclear cells. The pellet of granulocytes and remaining erythrocytes were subjected to a hypotonic lysis by exposure to 20 ml of ice-cold sterile for 30 seconds. The granulocytes were rescued with 20 ml of a 2x isotonic buffer (40 mM HEPES, 10 mM KCl, 10 mM D-glucose, 280 mM NaCl, pH 7.4) and pelleted. The lysis was repeated if erythrocytes remained. The specimen was then incubated with anti-CD-16-coupled immunomagnetic beads and processed as previously described (35). The resulting preparations all contained ≥ 98% eosinophils.

**Eosinophil production of oxidants and cyanate.** Purified eosinophils were washed, suspended at 4x10⁶/ml in a modified Hank’s balanced salt solution composed of 5.3 mM KCl, 138 mM NaCl, 0.4 mM potassium phosphate, 5.3 mM sodium phosphate, 5.5 mM D-glucose, 1.3 mM CaCl₂, and 0.5 mM MgCl₂, pH 7.4. supplemented with 1 mM KSCN. Triplicate groups were then supplemented with nothing (control), 1 µg/ml phorbol 12-myristate 13-acetate (PMA), or 1 µg/ml PMA and 5 mM azide, then incubated at 37°C for 60 minutes in capless 5 ml round-bottom tubes. The tubes were vortexed every 15 minutes to aid oxygenation. After the incubation, the samples were centrifuged for 10 minutes, 1000 x g at 4°C. The supernatants were placed in Microcon concentrators (Amicon, MA) with membranes of 3000 Dalton molecular weight cut off and centrifuged for 30 minutes, 13,000 x g at 4°C to remove degranulated proteins. The filtrates were assayed for HOSCN and cyanate quantities as described above.
**OSCN⁻/OCN⁻ effects upon RBC glutathione and enzymes.** Human RBCs were suspended at a hematocrit of 2% in PBS, pH 7.4) supplemented with increasing concentrations of OSCN⁻/OCN⁻ generated as above by the EPO/SCN⁻/H₂O₂ system. After 30 min, cells were pelleted by centrifugation at 2000 x g 3 min, and washed three times in ice-cold H/H buffer. Under these conditions, only at ≥ 200 µM OSCN⁻/OCN⁻ was there detectable hemolysis as assayed by spectrophotometric assay of supernatant hemoglobin or methemoglobin. RBC lysates and membranes were then prepared by hypotonic lysis and assayed for glutathione and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione-S-transferase (GST), and lactate dehydrogenase (LDH) activity as described by Beutler (53) and for ATPase activity as described below. Hemolysates were incubated 15 min further either with or without 10 mM DTT and reassayed for GAPDH, GST, and ATPases.

**Comparative oxidant inactivation of red blood cell ATPase activity.** Human red blood cell (RBC) membranes were prepared by hypotonic lysis. RBC membranes were suspended at 0.2 mg protein/ml in membrane buffer [20 mM Hepes, 5.5 mM NaCl, with 1g/L D-glucose, 0.10g/L MgCl₂• 6H₂O, 0.10 g/L MgSO₄• 7 H₂O, and 0.4 g/L KCl, pH 7.4] supplemented with increasing concentrations of H₂O₂, HOCl [diluted from concentrated hypochlorite (Sigma)], HOBr [prepared adding excess bromide to stock HOCl, as previously described (62)] or OSCN⁻/OCN⁻ (prepared by EPO as described above). RBC membranes were incubated with the oxidants for 15 min at 37 °C then pelleted and washed three times in ATPase buffer without oxidant. Total (i.e., Ca²⁺-, Mg²⁺-, calmodulin- and Na⁺/K⁺- dependent) ATPase activity was assayed in 0.2 mg protein RBC membrane aliquots suspended in 500 µl ATPase buffer (90 mM histidine, 90 mM imidazole, 15 mM MgCl₂, 400 mM NaCl₂, 0.5 mM EGTA, 75 mM KCl, 1 mM Ca Cl₂, 300 mM calmodulin, pH 7.4) by adding 3 mM ATP, incubating 10 min at 37 °C, then quenching phosphate release by adding 1000 µl 1% ascorbate in 10% trichloroacetic acid. Phosphate release was quantitated as described (63) by addition of 250 µl 1% ammonium molybdate tetrahydrate, incubating 5 min, then adding 500 µl 2% sodium citrate dihydrate and 2% sodium arsenite in 2% acetic acid. The resulting solution was assayed with a spectrophotometer at
700 nm and phosphate calculated from a standard curve generated using H₂KPO₄. Values were corrected for both spontaneous membrane phosphate release (i.e., membranes without ATP added) and spontaneous ATP hydrolysis (i.e., ATP in the absence of membranes).

**PAGE gels of GST exposed to [¹⁴C] OSCN⁻/OCN⁻:** 10 µg of human GST-π was exposed to 200µM [¹⁴C] OSCN⁻/OCN⁻ (generated using [¹⁴C] SCN⁻ in the EPO/SCN/H₂O₂ system described above) or H/H buffer. Samples were suspended in Laemmli buffer with or without β-mercaptoethanol (BME) and separated on a 3-15% SDS/PAGE gel (64). Gels were stained with Coomassie Blue and autoradiograms developed by fluorographically enhanced exposure of X-ray film (Kodak X-AR).
RESULTS

*Ph and temperature-dependence of EPO/SCN/H₂O₂ system oxidants.* To characterize the oxidant reaction product(s) generated by the EPO/SCN⁻/H₂O₂ system we assayed TNB-titratable oxidant stability over time as a function of pH and temperature (Figure 1). Under the conditions employed, more total oxidant is initially generated at pH 6.0 than at pH 7.4 (350 µM vs. 275 µmol). When subsequently incubated either at 25°C or 4°C, at pH 7.4 the oxidant half-life was more than six hours at 4°C and 60 minutes at 25°C. At pH 6.0 the half-life was approximately four hours at 4°C but less than 30 minutes at 25°C. Thus, oxidant generation by the EPO/SCN⁻/H₂O₂ system is favored at acidic versus physiologic pH; in contrast, oxidant stability is favored at 4°C and physiologic pH. These stability characteristics are similar to those of the oxidant product of the lactoperoxidase/SCN⁻/H₂O₂ system (22,25,26).

*NMR analysis of EPO/SCN/H₂O₂ system reaction products.* To enumerate and characterize the major products -- whether oxidant or non-oxidant -- of the EPO/SCN/H₂O₂ system, we employed [¹³C] NMR analysis of solutions containing [¹³C] SCN⁻ substrate (Figure 2). Analysis was performed both at pH 7.4 (*panels A, B, and C*) and pH 6.0 (*panels D and E*). At pH 7.4, spectra accumulated over the first 90 minutes show the expected large parent [¹³C] SCN⁻ resonance (*a*) at 133.4 PPM. In addition, two new peaks are discernible: (*b*) at 128.6 PPM along with a more intense resonance (*c*) at 127.3 PPM. These two latter peaks were absent from the NMR spectrum of [¹³C] SCN⁻ alone or of [¹³C] SCN⁻ in the presence of added H₂O₂ without EPO (*panel A*), and are therefore EPO-dependent. Both products are still detectable after incubation overnight at 4°C at pH 7.4 (*panel C*). In contrast, at pH 6.0 (*panels D and E*) although the first spectrum shows peaks *b* and *c* (*panel D*), after overnight incubation, peak *b* remains detectable, but peak *c* has disappeared and a new peak, *d*, is seen at 124.6 ppm. Thus, peak *c* has a pH- and temperature-stability profile compatible with that of the TNB-titratable oxidant depicted in Figure 1. By contrast, peak *b* is relatively stable, at least at 4°C. Peak *d*, which appears coincident with the
disappearance of peak c, may represent a decomposition product of peak c. There are therefore two major initial stable reaction products (i.e., peaks b and c) of the EPO/SCN⁻/H₂O₂ system.

**Mass spectrometry analysis of EPO/SCN⁻/H₂O₂ system reaction products.** Although these NMR studies suggest there are two major stable products of the EPO/SCN⁻/H₂O₂ system, they do not identify their structures. Moreover, because of the relative insensitivity of this technique, they do not rule out the possibility that other products exist. We therefore used electrospray ionization mass spectometry (ESI-MS) to analyze the reaction mixture (Figure 3). To confirm that putative reaction products resulted from oxidation of SCN⁻, we employed both [¹²C] SCN⁻ and [¹³C] SCN⁻ as substrates and sought the predicted isotope shift in the resultant carbon-containing products. Prior to addition of H₂O₂ to EPO and SCN⁻, a large parent SCN⁻ ion peak with m/z 58 was seen (not shown). Panel A shows the ESI-MS spectrum of an acetate buffer, H₂O₂, and SCN⁻ control solution in the absence of EPO. A large contaminant ion is seen at m/z 77 as are several smaller ions, including two with m/z 74 and 75. In the presence of the complete EPO/SCN⁻/H₂O₂ system using [¹²C] SCN⁻ (panel B) an ion with m/z 74 peak is formed, consistent with generation of OSCN⁻. When [¹³C] SCN⁻ was substituted for [¹²C] SCN⁻ in the complete EPO/SCN⁻/H₂O₂ system (panel C), no significant ion with at m/z 74 is generated [i.e., ion intensity is the same as in the absence of EPO (panel A)], but there is a large new ion at m/z 75, consistent with formation of [¹³C] OSCN⁻. These results are compatible with OSCN⁻ (m/z 74) being one major product of the EPO/SCN⁻/H₂O₂ system.

In scanning the range m/z 10-180 the only other new peak developing in the presence of the EPO/SCN⁻/H₂O₂ system was at m/z = 42 (Figure 3, panels D, E). In the acetate buffer, H₂O₂, and SCN⁻ control in the absence of EPO (panel D, only a low intensity (i.e., background) ion at m/z 42 is seen. An ion with m/z 42 is generated by the EPO/SCN⁻/H₂O₂ system using [¹²C] SCN⁻, consistent with the formation of [¹²C] OCN⁻. Confirming that this ion arises from SCN⁻, substitution of [¹³C] SCN⁻ for [¹²C] SCN⁻ yields a prominent new ion with m/z 43 instead of 42 (panel F). Under these conditions, the m/z 42 ion intensity is the same as in the control lacking EPO (panel D), indicating that this background m/z 42 ion is likely a contaminant in our buffer.
system. Based on these results we propose that OCN\(^{-}\) (m/z 42) is the second major product of the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system.

However, ESI-MS analyses at low m/z range are difficult because it is extremely difficult to get rid of all background ions in this low mass range. Moreover, a number of potential compounds have a m/z of 74 and 42. To characterize these compounds further, we therefore employed collision-induced dissociation tandem mass spectrometric analysis (MS/MS). As shown in Figure 4, panel A, when \([^{12}\text{C}]\) SCN\(^{-}\) was the substrate, the parent ion with m/z 74 fragmented to yield a predominant daughter ion of m/z 26 (compatible with CN\(^{-}\)), as well as less abundant ions at m/z 58 (compatible with SCN\(^{-}\)) and m/z 42 (compatible with OCN\(^{-}\)). Similarly, the m/z 42 ion also fragmented, though less extensively, to yield a daughter ion of m/z 26 (panel B). Parallel MS/MS studies ions at m/z 75 and at m/z 43 arising from substitution of \([^{13}\text{C}]\) SCN\(^{-}\) for \([^{12}\text{C}]\) SCN\(^{-}\) both yielded daughter ions consistent with these assignments (not shown). Collectively, these data strongly suggest that the ions representing the major catalysis products of the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system are attributable to OSCN\(^{-}\) and OCN\(^{-}\).

**Quantitative detection of OSCN\(^{-}\) and cyanate as major products of both the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system and activated human eosinophils: kinetic relationship between OSCN\(^{-}\) and OCN\(^{-}\) generation.** Detection of an ion with m/z compatible with OCN\(^{-}\) by mass spectrometry could represent an artifact of OSCN\(^{-}\) fragmentation during the ionization process. Therefore, to ascertain whether OCN\(^{-}\) is present in the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system reaction product prior to mass spectrometry analysis, we used a well-characterized analytical assay based upon the reaction of OCN\(^{-}\) with 2-aminobenzoic acid and subsequent cyclization in acid conditions to form 2,4 (1H,3H)-quinazolinedione, which is detected at 310 nm (33). In experiments not shown, we first showed that this assay for OCN\(^{-}\) did not also detect HOSCN by selectively titrating the reaction mixture with dithiothreitol, confirming absence of HOSCN by ESI-MS, and showing that the OCN\(^{-}\) assay detected similar amounts of OCN\(^{-}\) before and after the selective depletion of HOSCN.
Using this assay, we find that the EPO/SCN\(^-\)/H\(_2\)O\(_2\) system generates large amounts of cyanate with a time course that lags behind that of OSCN\(^-\). Figure 5 shows simultaneous determinations of oxidant (as TNB-titratable material) and OCN\(^-\) (as determined in the aminobenzoic acid reaction) at various time-points during the progression of the EPO/SCN\(^-\)/H\(_2\)O\(_2\) reaction. H\(_2\)O\(_2\) was continuously infused at 50 µM/min for 20 min, simulating the continuous generation of H\(_2\)O\(_2\) by the respiratory burst of an activated eosinophil. Aliquots were removed from the reaction mixture at one-minute intervals and catalase was added to destroy excess unreacted H\(_2\)O\(_2\) prior to assay of TNB-reactive substances and cyanate. OCN\(^-\) generation initially "lags" behind that of HOSCN for 5 minutes. HOSCN production plateaus at \(\approx 175\) µM while cyanate accumulation continues only so long as H\(_2\)O\(_2\) infusion continues (i.e., 20 min) then abruptly ceases thereafter. **This plateauing of HOSCN levels during H\(_2\)O\(_2\) infusion reflects a balance between rapid generation by the EPO and consumption by some secondary reaction, because addition of the EPO inhibitor azide at this stage of the progression curve causes an abrupt collapse of HOSCN levels (not shown).** Taken together, these data suggest that HOSCN is the initial product of the EPO/SCN\(^-\)/H\(_2\)O\(_2\) system and that OCN\(^-\) results from a subsequent reaction of HOSCN—e.g., reaction with excess H\(_2\)O\(_2\), reaction with a second molecule of HOSCN, or spontaneous decomposition.

To determine which of the NMR resonance peaks (i.e., \(b\) or \(c\)) detected in Figure 2 represents OCN\(^-\), we analyzed the NMR spectrum of \([^{13}\text{C}]\) urea. Although \([^{13}\text{C}]\) OCN\(^-\) is not commercially available, urea exists in equilibrium with small (approximately 0.5% at physiologic pH) amounts of cyanate (44). The NMR spectrum of \([^{13}\text{C}]\) urea at pH 7.4 (Figure 6) shows the expected large main urea peak at 162.5 PPM (36) as well as a smaller OCN\(^-\) peak at 128.6 PPM. The shift of this peak is indistinguishable from that of peak \(b\) in Figure 2, supporting its identification as OCN\(^-\). Since sulfur is less electron withdrawing than oxygen, the upfield resonance (peak \(c\) in Figure 2) is likely \([^{13}\text{C}]\) OSCN\(^-\).

To rule out non-enzymatic oxidation of SCN\(^-\) by H\(_2\)O\(_2\) as the basis for OCN\(^-\) generation by the EPO/SCN\(^-\)/H\(_2\)O\(_2\) system we either omitted EPO or, alternatively, blocked EPO catalytic activity
with the potent inhibitor azide (Figure 7.) Under both conditions, generation of both HOSCN and OCN⁻ were nearly completely blocked suggesting that generation of OCN⁻ as well as HOSCN depend upon the catalytic action of EPO.

As a gauge of the potential physiologic relevance of the above findings— all made with a purified reagent system— we determined whether intact, activated human eosinophils also generate HOSCN and OCN⁻ (Figure 8). Peripheral blood eosinophils were isolated from cancer patients undergoing experimental therapy with subcutaneous injections of interleukin-2, a circumstance known to be associated with the secondary elevation of blood interleukin-5 with consequent eosinophilia and activation of circulating eosinophils (37). Eosinophils isolated from such patients spontaneously generated low but detectable amounts of HOSCN and OCN⁻ (left pair of bars). When maximally stimulated by addition of phorbol myristate acetate, eosinophils generated greatly increased and roughly equimolar amounts of both HOSCN and OCN⁻ (middle pair of bars). Addition of 5 mM azide severely attenuated phorbol myristate acetate-stimulated generation of both products, though HOSCN more so than OCN⁻. Thus, activated human eosinophils generate both HOSCN and OCN⁻ by an EPO-dependent mechanism.

Because the products of the lactoperoxidase/H₂O₂/SCN⁻ system have been shown to react almost exclusively with SH groups (see Discussion below), we asked whether the EPO/SCN⁻/H₂O₂ system products react similarly and inflict SH-based toxicity. As a model we employed intact human red blood cells (RBCs) exposed to increasing concentrations of OSCN⁻/OCN⁻ generated by the EPO/SCN⁻/H₂O₂ system and assayed intracellular levels of the SH-containing oxidant scavenger glutathione. In addition, we assayed the activities of three enzymes [glutathione-S-transferase (GST), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and membrane ATPases] which are known (66-68) to be vulnerable to SH-oxidation-based inactivation, as well as one, lactate dehydrogenase (LDH), not prone to such inactivation (Figure 9). Exposure of RBCs to OSCN⁻/OCN⁻ under these conditions produced no detectable hemolysis. With increasing concentrations of OSCN⁻/OCN⁻, glutathione is first depleted, followed by inactivation of GST and GAPDH, then membrane ATPases. LDH activity was completely unaffected. This inactivation of
GST, GAPDH, and ATPase activity is based on reversible SH oxidation because subsequent treatment of lysates and membranes prepared from RBCs exposed to 100 µM OSCN⁻/OCN⁻ with 10 mM dithiothreitol (DTT), a SH reducing agent, completely restores the activities of all three enzymes (not shown). In aggregate, these findings suggest that EPO/SCN⁻/H₂O₂ products diffuse through intact RBC membranes to oxidize intracellular SH-containing compounds.

Given the potential specificity of OSCN⁻/OCN⁻ for SH reactivity, we hypothesized that these substances, despite being far less reactive for other biologic materials than the potent bleaching oxidants HOCl and HOBr, might more effectively mediate SH-based toxicity. We therefore compared the capacity of these oxidants as well as H₂O₂ to inactivate ATPases in isolated RBC membranes (Figure 10). Membrane preparations rather than intact RBC were used to allow all oxidants equal access to intra- and extracellular membrane components. In this model OSCN⁻/OCN⁻ inhibits RBC ATPases much more effectively than in intact RBCs (cf. Figure 9) with an ID₅₀ of 2 µM, 1/10th that of HOCl and HOBr; H₂O₂ fails to inactivate ATPases even at 1 mM. Thus, the failure of OSCN⁻/OCN⁻ to react indiscriminantly with a wide array of other membrane components allows it to function more effectively than do more potent oxidants as a SH-based metabolic inhibitor.

To examine the structural basis of SH-dependent inactivation of GST by OSCN⁻/OCN⁻, we exposed human GST-π, the isoform found in RBCs, to [¹⁴C] OSCN⁻/OCN⁻. Proteins were then analyzed by SDS/PAGE for electrophoretic mobility and [¹⁴C] incorporation under reducing and non-reducing conditions (Figure 11). As shown in the left panel (lanes 1-4), GST not exposed to [¹⁴C] OSCN⁻/OCN⁻ runs as a single band (lane 1) with a mobility of 23 kD under reducing conditions; under non-reducing conditions (lane 2) an additional faint band of higher mobility, presumably representing a species containing intramolecular disulfide bonds, is evident. After exposure to [¹⁴C] OSCN⁻/OCN⁻, under nonreducing conditions (lane 4), the 23 kD is reduced in intensity, that of the higher mobility band increased, and an additional faint band of ca. 48 kD mobility is also seen. That this last band represents disulfide-bonded intermolecular dimers is shown by the fact that it, along with the high mobility band, disappear upon reduction (lane 3).
Autoradiograms of lanes 3 and 4 (right panel, lanes 3A and 4A), show heavy, nearly completely SH reductant-reversible of $[^{14}\text{C}]$ into all three of these bands and a faint band of even higher mobility. Thus, exposure of GST to $[^{14}\text{C}]\text{OSCN}^-/\text{OCN}^-$ causes intra- and intermolecular disulfide bond formation, as well as SH reductant-reversible bonding of a $[^{14}\text{C}]$–containing moiety.

**DISCUSSION**

*Products of the EPO/SCN$^-$/H$_2$O$_2$ system.* Peroxidase-catalyzed oxidation of SCN$^-$ has hitherto been best characterized in the case of lactoperoxidase, an enzyme present in large quantities in milk, saliva, and tears. Lactoperoxidase has extensive structural and therefore presumably functional similarities to EPO based upon primary amino acid sequence (1,2,8) and prosthetic group resonance characteristics (54-57). SCN$^-$, termed a pseudohalide because it reacts as a halide in several peroxidase-catalyzed reactions (25), is present in human serum at concentrations of 10-100 µM (25). Serum SCN$^-$ derives from a detoxification reaction between cyanide and thiosulfates and, more importantly, from ingestion of SCN$^-$ and related compounds from dietary sources (25). In saliva, milk, and tears, SCN$^-$ concentrations reach millimolar levels and SCN$^-$ is the principal substrate for lactoperoxidase. The weak oxidant products of the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system are bacteriostatic and bactericidal. By this mechanism the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system appears to play a critical role in preventing bacterial overgrowth in the oral cavity, gut, and eyes (25,38). In addition, the product(s) of this system are cytocidal for candida fungus (58) and certain viruses (59) but relatively innocuous for mammalian cells (27).

The predominant oxidative product(s) of this reaction has been suggested, by analogy with the hypohalous acids HOCl and HOBr, to be hypothiocyanous acid (HOSCN) (22,25,26). However, efforts to identify definitively the species produced by the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system have been hampered by the inability to produce authentic HOSCN by chemical synthesis (25). Modi et al. characterized the products of this system using $[^{15}\text{N}]$ SCN$^-$ NMR (29).
These studies demonstrated the formation of one major reaction product with a resonance of 179 PPM. However, in the presence of excess H$_2$O$_2$, this peak disappeared and a new one at 128 PPM appeared, which they attributed to CN$. However, in contrast, Pollock and Goff, using $^{13}$C-labeled SCN$, found (28) that the initial stable reaction product of this reaction appeared at 126.5 PPM, which they attributed to dicyanate ether (NCS-O-SCN). Twenty to 30 minutes later this initial product peak declined coincident with the appearance of a new species at 128.6 PPM, which they attributed to OSCN$. Lövaas (30), based on ESR studies of the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system, suggested that the major stable product of the reaction was ‘OSC*N’. Thus, spectral analysis of the products of the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system have resulted in three different species other than HOSCN being proposed as the initial reaction product.

We now present evidence that the major products of the EPO/SCN$^-$/H$_2$O$_2$ system are OSCN$^-$ and OCN$. Preliminary experiments using ESI-MS suggest that OSCN$^-$ and OCN$^-$ are also the predominant products of the lactoperoxidase/SCN$^-$/H$_2$O$_2$ system as well (not shown). We therefore predict that the two major product resonance peaks previously detected by $^{15}$N SCN$^-$ NMR (29) and $^{13}$C OCN$^-$ NMR (28) studies of this system will also prove to be attributable to OSCN$^-$ and OCN$. If so, both EPO- and lactoperoxidase-mediated oxidation of their principal physiologic substrate, SCN$, by H$_2$O$_2$ yields two compounds with limited but specific reactivities (see below).

**Mechanism of HOSCN and OCN$^-$ generation by the EPO/SCN$^-$/H$_2$O$_2$ system.** Our data suggest, but do not prove, that the EPO/SCN$^-$/H$_2$O$_2$ system first generates HOSCN, which then subsequently reacts with excess H$_2$O$_2$, a second molecule of HOSCN, or decomposes to generate OCN$^-$ secondarily. The first possibility is supported by Figure 5, in which generation of OCN$^-$ clearly "lags" behind generation of HOSCN, which plateaus at ca. 150 μM while OCN$^-$ accumulation continues, but only so long as H$_2$O$_2$ is being added. A reaction sequence that follows this scenario has been proposed by Aune *et al.* and Thomas (25,26):

$$
\text{Eq. 1} \quad \text{H}_2\text{O}_2 + \text{SCN}^- + \text{H}^+ \rightarrow \text{HOSCN} + \text{H}_2\text{O}
$$

$$
\text{Eq. 2} \quad \text{H}_2\text{O}_2 + \text{HOSCN} \rightarrow \text{HOOSCN} + \text{H}_2\text{O}
$$
Eq. 3  \[ \text{HOOSCN} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{SO}_3 + \text{HOCN} \]

A number of other mechanisms are conceivable. Because our experiments were designed to detect relatively stable products of the EPO/SCN⁻/H₂O₂ system, they do not rule in or out the transient existence of what are likely highly reactive and unstable intermediates such as HOOSCN (cyanosulfurous acid).

**EPO/SCN⁻/H₂O₂ as a sulfhydryl reactivity-based cytotoxic system.** In a model cytotoxic system employing human RBCs, we find that graded exposure to the products of the EPO/SCN⁻/H₂O₂ system first depletes glutathione, then causes SH-reductant reversible inactivation of GAPDH and GST, then membrane ATPases without detectable hemolysis (Figures 9 and 10). This sequence suggests a mechanism of toxicity based largely, if not exclusively, upon SH reactivity of a membrane-permeant, diffusible agent. This pattern, also seen with the products of the lactoperoxidase/SCN⁻/H₂O₂ system (40), contrasts strikingly with that of HOCl, which reacts rapidly with a variety of cell surface biologic targets, rapidly disrupting membrane integrity and lysing cells prior to oxidizing intracellular proteins to a significant degree (3-5). A weak, but relatively SH-specific reactive oxidant such as HOSCN can more effectively function as a SH-dependent metabolic poison than a potent, but non-specifically reactive oxidant such as HOCl (Figure 10).

Potential mechanisms underlying such toxicity are suggested using GST-\(\pi\) as a model protein target (Figure 11), where it is shown that \[^{14}\text{C}\text{OSCN}⁻/\text{OCN}⁻\] causes intra- and intermolecular disulfide bonds and, in addition, covalent incorporation of \[^{14}\text{C}\] that is nearly completely reversible by SH reductants. \[^{14}\text{C}\] incorporation may reflect sulfenyl thiocyanate bond formation (R-S-SCN), which can either persist, be reversed by a SH reductant, or react further with another SH to yield a disulfide: R-S-SCN + R'SH = RSSR' + SCN⁻ + H⁺ (25). \[^{14}\text{C}\] incorporation could theoretically also reflect carbamoylation reactions of OCN⁻ with SH groups (see below).

What, if any, contribution OCN⁻ makes to the cytotoxicity of the EPO/SCN⁻/H₂O₂ system remains to be determined. It may simply be an inert decomposition product of HOSCN. However,
the large amounts of OCN\(^-\) produced by the EPO/SCN\(^-\)/H\(_2\)O\(_2\) system and the known chemical reactivity and toxicity of OCN\(^-\) raise the possibility -- not addressed by our experiments-- that it could play a role by modifying target proteins. Unlike the electrophile oxidants HOCl and HOSC\(_2\), OCN\(^-\) is a nucleophile that reacts irreversibly with amino groups and slowly, but reversibly with thiol groups of proteins through carbamoylation reactions (41). Thus, OCN\(^-\) has been shown to react with \(\alpha\)-amino, \(\varepsilon\)-amino, and thiol moieties of amino acids, peptides and proteins (41). These carbamoylation reactions can irreversibly inactivate enzymes such as G6PD (41,42). Moreover, OCN\(^-\) can directly react with glutathione (41), and RBCs exposed to OCN\(^-\) have decreased levels of intracellular glutathione (43). An alternative potential role for OCN\(^-\) is suggested by the finding that 100-1000 \(\mu\)M OCN\(^-\) strongly inhibits MPO generation of HOCl by an H\(_2\)O\(_2\)-dependent mechanism suggesting autoinactivation (46). We find that OCN\(^-\) also inhibits HOSC\(_2\) production by EPO, but only at concentrations \(\geq 1\) mM (not shown). Despite these possibilities, however, our data showing SH-reactivity based toxicity can be accounted for solely by the proposed oxidant reactivity of OSCN\(^-\) without invoking a role for OCN\(^-\), because OCN\(^-\) reaction with SH groups is slow in comparison with that of OSCN\(^-\) (41). On the other hand, OCN\(^-\), unlike OSCN\(^-\), reacts rapidly with amino groups (41). Experiments with selective scavenging agents may permit dissection of the contribution of each of these species to cytotoxicity.

**Potential physiologic significance of findings.** Both OSCN\(^-\) and OCN\(^-\) have relatively specific reactivities that may bespeak a novel biologic strategy for the EPO system in carrying out the putative role of eosinophils, i.e., extracellular destruction of metazoan parasites (6). In preliminary studies we have found (61) that the ED\(_{50}\) for killing of cultured human and mammalian cells --including cardiac myocytes and several types of endothelial cells-- is approximately 10-fold higher (200-300 \(\mu\)M oxidant) for EPO/H\(_2\)O\(_2\)/SCN\(^-\) system-derived products than for HOCl or HOBr; however, the ED\(_{50}\) for killing of freshly transformed schistosomules, a model of parasitic disease, is nearly identical (20-30 \(\mu\)M oxidant). It is therefore conceivable that OSCN\(^-\) and OCN\(^-\) comprise a cytotoxic system that exploits subtle, sulfhydryl-based metabolic differences between host and parasites to effect selective killing of the latter. These weakly, but
specifically, reactive compounds may be better suited for selective extracellular killing of pathogens than is a potent but nonspecific oxidant such as HOCl. Supporting the plausibility of a sulfhydryl-based cytotoxic system, the efficacy of several anti-parasite drugs, such as oltipraz (47) and certain arsenicals (48), derives from selective oxidative depletion and impaired regeneration of parasite glutathione, a compound also readily oxidized by both OSCN\(^{-}\) and OCN\(^{-}\). Our findings also have implications for the potential role of EPO in the pathogenesis of human diseases, such as asthma and other allergic states, in which EOs are known to play an important role. Covalent modification of crucial cellular or extracellular SH-containing biological targets by the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system, as we have shown to occur in RBCs, could contribute to the initiation and propagation of the host tissue damage. Carbamoylation of host cell amine-containing protein and lipid targets in the setting of chronic eosinophilic inflammatory states could play a similar role. Identification and quantitation of stable OSCN\(^{-}\) or OCN\(^{-}\) modified biologic targets containing SH or amino groups might serve as a sensitive and specific marker of the EPO/SCN\(^{-}\)/H\(_2\)O\(_2\) system’s participation in these diseases. Given these possibilities, the potential roles of OSCN\(^{-}/\)OCN\(^{-}\) as mediators of eosinophil-mediated toxicity, both for pathogens and host tissue, warrants further scrutiny.
Figure Legends

FIGURE 1. pH- and temperature-dependence of oxidants generated by the EPO/SCN⁻/H₂O₂ system. Five consecutive 250 µM increments of H₂O₂ were added at one minute intervals to 1 ml of a solution containing 2 mM KSCN and 0.4 µM EPO in 0.1 M sodium phosphate buffer at either pH 6.0 (boxes) or 7.4 (triangles) at 25°C. One minute after addition of the final bolus, 20 µl of catalase (2 mg/ml) was added to consume excess unreacted H₂O₂. After addition of catalase, reaction mixtures were incubated either at 25°C or 4°C and aliquots sampled at the indicated time intervals for total TNB titratable oxidants. ■ pH 6.0, 25°C; □ pH 6.0, 4°C; ▲ pH 7.4, 25°C; △ pH 7.4, 4°C.

FIGURE 2. [¹³C] SCN⁻ NMR analysis of EPO/SCN⁻/H₂O₂ system reaction products. Using the protocol described for Figure 1, reaction products of the EPO/SCN⁻/H₂O₂ system were generated either at pH 7.4 (panels A, B, and C) or pH 6.0 (panels E and F) at room temperature, then maintained thereafter at 4°C for subsequent evaluation of products and their stabilities. Reaction mixtures were generated and NMR spectra obtained in buffers supplemented with 10% D₂O. Spectra (1500-2000) were obtained using a Bruker AMX-600 using an 8 mm broad-band probe over 90-120 min. Chemical shifts were referenced relative to an external (CH₃)₄Si standard. Panel A: pH 7.4, SCN⁻/H₂O₂ as described in legend for Figure 1 without EPO. 10,000 scans obtained to maximize sensitivity. Panel B: pH 7.4. Reaction products of the EPO/SCN⁻/H₂O₂ system analyzed over first 90 min after generation; Panel C: pH 7.4. Reaction products from Panel B after incubation overnight (16h) at 4°C. Panel D: pH 6.0. Reaction products of the EPO/SCN⁻/H₂O₂ system analyzed over first 90 min after generation. Panel E: pH 6.0. Reaction products from Panel B after incubation overnight (16h) at 4°C. Peak assignments, based on our subsequently described studies and previous NMR studies (24,28), are: a = SCN⁻; b = OCN⁻; c = OSCN⁻; d = CO₂.
FIGURE 3. Negative ion electro spray ion mass spectrometry (ESI-MS) analysis of the EPO/SCN/H₂O₂ system reaction products. EPO/SCN⁻/H₂O₂ reaction products were generated at pH 7.4 in 10 mM ammonium acetate buffer supplemented with 0.4 µM EPO and 2 mM potassium thiocyanate, either [¹²C] SCN⁻ or [¹³C] SCN. Five consecutive 200 µM increments of H₂O₂ were added at one minute intervals to 1 ml of a solution containing 2 mM KSCN and 0.4 µM EPO in 0.1 M sodium phosphate buffer at either pH 6.0 (boxes) or 7.4 (triangles) at 25°C. One minute after the addition of the last bolus of H₂O₂, catalase (40 µg/ml) was added to consume unreacted excess H₂O₂. A syringe pump was then used to spray solutions at a flow rate of 5-10 µl/min into the SCIEX API III triple quadrupole mass spectrometer. Results are expressed as relative intensity as percent that of the largest peak in the m/z 10-180 spectrum, which in all cases was the [¹²C] or [¹³C] thiocyanate ion, m/z 58 or m/z 59, respectively. Upper panels (A, B, and C): m/z range 70-80. Panel A: control comprised of complete system as described above except lacking EPO; Panel B: complete system in the presence of [¹²C] SCN⁻; Panel C: complete system in the presence of [¹³C] SCN⁻. Lower panels (D, E, and F): m/z range 40-50. Panel D: complete system lacking EPO; Panel E: complete system in presence of [¹²C] SCN⁻; Panel F: complete system in presence of [¹³C] SCN⁻. *: contaminant peaks in areas of interest not dependent upon presence of EPO.

FIGURE 4. Collision-induced dissociation tandem mass spectrometry (MS/MS) analysis of the major EPO/SCN⁻/H₂O₂ system reaction products. The complete EPO/SCN⁻/H₂O₂ system was run in the presence of [¹²C] SCN⁻ as described in the legend for Figure 3. Panel A: MS/MS spectrum of m/z 74 parent ion. Panel B: MS/MS spectrum of m/z 42 parent ion. Ion assignments are indicated in brackets.

FIGURE 5. Time course of OSCN⁻ and cyanate production by the EPO/SCN⁻/H₂O₂. To solutions containing 0.4 µM EPO, 1 mM KSCN in potassium phosphate
buffer 15 mM, pH 7.4, \( \text{H}_2\text{O}_2 \) was added by continuous infusion of 50 \( \mu \text{M/min} \) over 20 minutes, then discontinued. At the indicated time points aliquots were withdrawn, supplemented with 40 \( \mu \text{g/ml catalase} \) to consume excess unreacted \( \text{H}_2\text{O}_2 \), placed on ice, and subsequently assayed for oxidant (■) by TNB titration and cyanate (□) by derivativization of 2-aminobenzoic acid (see Methods). Reactions took place at room temperature.

**FIGURE 6. \([^{13}\text{C}]\) Urea NMR analysis to establish shift of OCN\(^-\).** A 1 M solution of \([^{13}\text{C}]\)-labeled urea was prepared in a PBS buffer (pH 7.4) supplemented with 10\% \( \text{D}_2\text{O} \) and incubated for 40 minutes in a water bath at 85\(^\circ\)C to promote equilibration with OCN\(^-\). Concomitant assay of OCN\(^-\) by chemical analytic assay in this preparation showed a cyanate concentration of \( \approx \) 5 mM (not shown). After cooling on ice, this preparation was then analyzed by NMR as described in Materials and Methods. All peaks were referenced relative to a (CH\(_3\))\(_4\)Si standard. The large resonance at 162.5 PPM represents the parent urea peak. The only other major peak is observed at 128.6 PPM. NMR analysis of freshly prepared \([^{13}\text{C}]\) urea prior to heating did not show any significant feature at 128.6 PPM (not shown).

**FIGURE 7. Peroxidase activity dependence of OSCN\(^-\) and OCN\(^-\) production by the EPO/SCN/\( \text{H}_2\text{O}_2 \) system.** Solutions containing 0.4 \( \mu \text{M EPO}, 1 \text{mM KSCN}, 50 \text{mM potassium phosphate buffer (pH 7.4)} \) were supplemented with \( \text{H}_2\text{O}_2 \) (added as 100 \( \mu \text{M increments each minute x 5} \)), subsequent to which catalase (40 \( \mu \text{g/ml} \)) was added to consume excess unreacted \( \text{H}_2\text{O}_2 \). OSCN\(^-\) and OCN\(^-\) were subsequently determined in the presence of the complete system (left pair of bars); the complete system in the presence of the peroxidase inhibitor azide, 5 mM (middle pair of bars); or in a system lacking added EPO (right pair of bars). Data represent mean ± S.E.M. \( n=3 \) for each group.

**FIGURE 8. OSCN\(^-\) and OCN\(^-\) production by PMA-activated eosinophils.** Purified human eosinophils (4 \( \times \) 10\(^6\)/ml) isolated from three different donors were suspended in modified
H/H buffer (pH 7.4) supplemented with 1 mM KSCN. Cells were then incubated for 60 minutes at 37°C in the absence of further additions (left pair of bars), after addition of 100 ng/ml of PMA (center pair of bars), or in the concomitant presence of both 5 mM sodium azide and 100 ng/ml of PMA (right pair of bars). Supernatant buffer obtained by spinning 5000 x g for 5 minutes was deproteinated by Microcon concentrators (Amicon, MA) with membranes of 3000 kD molecular weight cut off and centrifugation for 30 minutes, 13,000 x g at 4°C. Filtrates were assayed for oxidants and cyanate as previously described.

FIGURE 9. Inactivation of RBC enzymes by products of the EPO/SCN-/H₂O₂ system. Human RBCs were suspended at a hematocrit of 2% in PBS buffer (pH 7.4) and the indicated concentration (titrated by TNB oxidation) of OSCN-, generated by the EPO/SCN-/H₂O₂ system using the protocol described in the legend for Figure 6, for 30 min at 37°C. After pelleting and washing 3 times rapidly in ice cold PBS buffer, lysates were prepared and assayed for GSH, GAPDH, GST and LDH; membrane preparations were assayed for ATPase activity. Under these conditions there was no detectable hemolysis.

FIGURE 10. Comparative oxidant inactivation of red blood cell membrane ATPases. 100 µg aliquots of RBC membranes were exposed to the indicated concentrations of OSCN- (titrated by TNB oxidation and generated by the EPO/SCN-/H₂O₂ system) or reagent HOBr, HOCl, or H₂O₂ in H/H buffer 15 min at 37°C, washed 3 times in ice cold H/H buffer, then assayed for total ATPase activity by quantitation of phosphate release as described in Methods.

FIGURE 11. SDS/PAGE analysis of human GST-π exposed to [¹⁴C] OSCN-/OCN. 10 µg of human GST-π was exposed to 200 µM [¹⁴C] OSCN-/OCN- (generated using [¹⁴C] SCN- in the EPO/SCN-/H₂O₂ system described above) or H/H buffer. Samples were suspended in Laemmli buffer with or without beta mercaptoethanol (BME) and separated on a 3-15% SDS/PAGE gel. Gels were stained with Coomassie Blue (left four lanes: 1-4) and
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**FIGURE 1. pH- and temperature-dependence of oxidants generated by the EPO/SCN\(^-\)/H\(_2\)O\(_2\) system.** Five 250 µM boluses of H\(_2\)O\(_2\) were added at 1 minute intervals to 1 ml of a solution containing 2 mM KSCN and 0.4 µM EPO in 0.1 M sodium phosphate buffer at either pH 6.0 (boxes) or 7.4 (triangles). One minute after addition of the final bolus, 20 µl of catalase (2 mg/ml) was added to consume excess unreacted H\(_2\)O\(_2\). After addition of catalase, reaction mixtures were incubated either at 37°C or 4°C and aliquots sampled at the indicated time intervals for total TNB titratable oxidants. ■pH 6.0, 25°C; □pH 6.0, 4°C; ▲pH 7.4, 25°C; Δ pH 7.4, 4°C.
FIGURE 2. [13C] SCN⁻ NMR analysis of EPO/SCN⁻/H₂O₂ system reaction products. Using the protocol described for Figure 1, reaction products of the EPO/SCN⁻/H₂O₂ system were generated either at pH 7.4 (panels A, B, and C) or pH 6.0 (panels E and F) at room temperature, then maintained thereafter at 4°C for subsequent evaluation of products and their stabilities. Reaction mixtures were generated and NMR spectra obtained in buffers supplemented with 10% D₂O. Spectra (1500-2000) were obtained using a Bruker AMX-600 using an 8 mm broadband probe over 90-120 min. Chemical shifts were referenced relative to an external (CH₃)₄Si standard. Panel A: pH 7.4, SCN⁻/H₂O₂ as described in legend for Figure 1 without EPO. 10,000 scans obtained to maximize sensitivity. Panel B: pH 7.4. Reaction products of the EPO/SCN⁻/H₂O₂ system analyzed over first 90 min after generation; Panel C: pH 7.4. Reaction products from Panel B after incubation overnight (16h) at 4°C. Panel D: pH 6.0. Reaction products of the EPO/SCN⁻/H₂O₂ system analyzed over first 90 min after generation. Panel E: pH 6.0. Reaction products from Panel B after incubation overnight (16h) at 4°C. Peak assignments, based on our subsequently described studies and previous NMR studies (24,28), are: a = SCN⁻; b = OCN⁻; c = OSCN⁻; d = CO₂.
FIGURE 3. Negative ion electrospray ion mass spectrometry (ESI-MS) analysis of the EPO/SCN/H$_2$O$_2$ system reaction products. EPO/SCN$^-$/H$_2$O$_2$ reaction products were generated at pH 7.4 in 10 mM ammonium acetate buffer supplemented with 0.4 µM EPO and 2 mM potassium thiocyanate, either [13C] SCN$^-$ or [15C] SCN. Five consecutive 200 µM increments of H$_2$O$_2$ were added at one minute intervals to 1 ml of a solution containing 2 mM KSCN and 0.4 µM EPO in 0.1 M sodium phosphate buffer at either pH 6.0 (boxes) or 7.4 (triangles) at 25°C. One minute after the addition of the last bolus of H$_2$O$_2$, catalase (40 µg/ml) was added to consume unreacted excess H$_2$O$_2$. A syringe pump was then used to spray solutions at a flow rate of 5-10 µl/min into the SCIEX API III triple quadrupole mass spectrometer. Results are expressed as relative intensity as percent that of the largest peak in the m/z 10-180 spectrum, which in all cases was the [12C] or [13C] thiocyanate ion, m/z 58 or m/z 59, respectively. Upper panels (A, B, and C): m/z range 70-80. Panel A: control comprised of complete system as described above except lacking EPO; Panel B: complete system in the presence of [13C] SCN$^-;$ Panel C: complete system in the presence of [13C] SCN$^-$. Lower panels (D, E, and F): m/z range 40-50. Panel D: complete system lacking EPO; Panel E: complete system in presence of [12C] SCN$^-;$ Panel F: complete system in presence of [13C] SCN$^-$. *: contaminant peaks in areas of interest not dependent upon presence of EPO.
FIGURE 4. Collision-induced dissociation analysis of the major EPO/SCN$^-$/H$_2$O$_2$ system reaction products using (MS/MS). The complete EPO/SCN$^-$/H$_2$O$_2$ system was run in the presence of $[^{13}$C] SCN$^-$ employing five 100 µM boluses of H$_2$O$_2$ as described in the legend for Figure 3. Panel A: m/z 74 parent ion spectrum. Panel B: m/z 42 parent ion spectrum.
FIGURE 5. Time course of OSCN⁻ and cyanate production by the EPO/SCN⁻/H₂O₂. To solutions containing 0.4 µM EPO, 1 mM KSCN in potassium phosphate buffer 15 mM, pH 7.4, H₂O₂ was added by continuous infusion of 50 µM/min over 20 minutes, then discontinued (Panel C). At the indicated time points aliquots were withdrawn, supplemented with 40 µg/ml catalase to consume excess unreacted H₂O₂, placed on ice, and subsequently assayed for oxidant (■) by TNB titration and cyanate (□) by derivativization of 2-aminobenzoic acid (see Methods). Reactions took place at room temperature.
FIGURE 6. Peroxidase activity dependence of HOSCN and cyanate production by the EPO/SCN⁻/H₂O₂ system. Solutions containing 0.4 µM EPO, 1 mM KSCN, 50 mM potassium phosphate buffer (pH 7.4) were supplemented with H₂O₂ (100 µM boluses each 1 minute apart x 5), subsequent to which catalase 40 µg/ml was added to consume excess unreacted H₂O₂. HOSCN and cyanate were subsequently determined in the presence of the complete system (left pair of bars), the complete system in the presence of the peroxidase inhibitor azide, 5-mM (middle pair of bars), or in a system lacking added EPO (right pair of bars).
FIGURE 7. \[^{13}\text{C}]\text{Urea NMR analysis to establish shift of OCN}^\text{-}.\] A 1 M solution of \[^{13}\text{C}\]-labeled urea was prepared in a PBS buffer (pH 7.4) supplemented with 10% D\(_2\)O and incubated for 40 minutes in a water bath at 85°C to promote equilibration with OCN\(^\text{-}\). Concomitant assay of OCN\(^\text{-}\) by chemical analytic assay in this preparation showed a cyanate concentration of \(\approx 5\) mM (not shown). After cooling on ice, this preparation was then analyzed by NMR as described in Materials and Methods. All peaks were referenced relative to a (CH\(_3\))\(_4\)Si standard. The large resonance at 162.5 PPM represents the parent urea peak. The only other major peak is observed at 128.6 PPM.
FIGURE 8. HOSCN and cyanate production by PMA-activated eosinophils. Purified human eosinophils (4 x 10^6/ml) isolated from three different donors were suspended in modified H/H buffer (pH 7.4) supplemented with 1 mM KSCN. Cells were then incubated for 60 minutes at 37°C in the absence of further additions (left pair of bars), after addition of 100 ng/ml of PMA (center pair of bars), or in the concomitant presence of both 5 mM sodium azide and 100 ng/ml of PMA (right pair of bars). Supernatant buffer obtained by spinning 5000 x g for 5 minutes was deproteinated by Microcon concentrators (Amicon, MA) with membranes of 3000 kD molecular weight cut off and centrifugation for 30 minutes, 13,000 x g at 4°C. Filtrates were assayed for oxidants and cyanate as previously described.
FIGURE 9. Inactivation of rbc enzymes by products of the EPO/SCN/H_2O_2 system. Human rbcs were suspended at a hematocrit of 2% in H/H buffer (pH 7.4) and the indicated concentration (titrated by TNB oxidation) of OSCN, generated by the EPO/SCN/H_2O_2 system using the protocol described in the legend for Figure 6, for 15 min at 37°C. After pelleting and washing 3 times rapidly in ice cold H/H buffer, lysates were prepared and assayed for GSH, GAPDH, GST and LDH; membrane preparations were assayed for ATPase activity. Under these conditions there was no detectable hemolysis.
FIGURE 10. Comparative oxidant inactivation of red blood cell membrane ATPases. 100 μg aliquots were exposed to the indicated concentrations of OSCN⁻ (titrated by TNB oxidation and generated by the EPO/SCN⁻/H₂O₂ system) or reagent HOBr, HOCl, or H₂O₂ in H/H buffer 15 min at 37°C, washed 3 times in ice cold H/H buffer, then assayed for total ATPase activity by quantitation of phosphate release as described in Methods.
FIGURE 11. SDS/PAGE analysis of human GST-π exposed to $[^{14}\text{C}]$ OSCN$^-$/OCN$^-$. 10 µg of human GST-π was exposed to 200 µM $[^{14}\text{C}]$ OSCN$^-$/OCN$^-$ (generated using $[^{14}\text{C}]$ SCN$^-$ in the EPO/SCN$^-$/H$_2$O$_2$ system described above) or H/H buffer. Samples were suspended in Laemmli buffer with or without beta mercaptoethanol (BME) and separated on a 3-15% SDS/PAGE gel (XX). Gels were stained with Coomassie Blue (left panel) and autoradiograms developed by fluorographically enhanced exposure of X-ray film (right panel). Left panel: lane 1: buffer + BME; lane 2: buffer without BME; lane 3: 200 µM $[^{14}\text{C}]$ OSCN$^-$/OCN$^- +$ BME; lane 4: 200 µM $[^{14}\text{C}]$ OSCN$^-$/OCN$^-$, no BME. Right panel: Autoradiograms of lanes 3 (3A) and 4 (4A).
Eosinophil peroxidase oxidation of thiocyanate: characterization of major reaction products and a potential sulphydryl-targeted cytotoxicity system
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