Eosinophil Peroxidase Catalyzes Bromination of Free Nucleosides and Double-Stranded DNA†

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Received August 17, 2000; Revised Manuscript Received November 7, 2000

ABSTRACT: Chronic parasitic infections are a major risk factor for cancer development in many underdeveloped countries. Oxidative damage of DNA may provide a mechanism linking these processes. Eosinophil recruitment is a hallmark of parasitic infections and many forms of cancer, and eosinophil peroxidase (EPO), a secreted hemoprotein, plays a central role in oxidant production by these cells. However, mechanisms through which EPO may facilitate DNA oxidation have not been fully characterized. Here, we show that EPO effectively uses plasma levels of bromide as a cosubstrate to brominate bases in nucleotides and double-stranded DNA, forming several stable novel brominated adducts. Products were characterized by HPLC with on-line UV spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS). Ring assignments for brominated purine bases as their 8-bromo adducts were identified by NMR spectroscopy. Using stable isotope dilution LC/ESI/MS/MS, we show that while guanine is the preferred purine targeted for bromination as a free nucleobase, 8-bromoadenine is the major purine oxidation product generated following exposure of double-stranded DNA to either HOBr or the EPO/H_2O_2/Br^- system. Bromination of nucleobases was inhibited by scavengers of hypohalous acids such as the thioether methionine, but not by a large molar excess of primary amines. Subsequently, *N*-monobromoamines were demonstrated to be effective brominating agents for both free nucleobases and adenine within intact DNA. A rationale for selective modification of adenine, but not guanine, in double-stranded DNA based upon stereochemical criteria is presented. Collectively, these results suggest that specific brominated DNA bases may serve as novel markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants.

There is a well-established link between chronic inflammation and the development of certain forms of cancer (*1*−*7*). For example, multiple chronic parasitic infections are causally linked to cancer development (e.g., *Schistosoma haematobium* and bladder cancer and *Opisthorcis viverrini* and cholangiocarcinoma) and represent a leading cause of cancer mortality in some underdeveloped countries (*7*−*10*). Eosinophilic infiltration is a hallmark of these disorders (*11*). Similarly, chronic hepatitis and forms of inflammatory bowel disease (ulcerative colitis) are associated with dramatic increases in the risk for development of specific cancers [i.e., hepatocellular carcinoma and adenocarcinoma of the colon, respectively (*11*)]. The mechanisms linking the associations between chronic inflammation and cancer development are unknown. However, the prodigious capacity of leukocytes to generate free radicals and reactive oxidant species (*12*−*15*), coupled with the significant data linking DNA oxidative damage to mutagenesis and cancer development (*16*−*20*), strongly suggests that leukocyte-dependent oxidative damage of DNA may be a mechanism.

During leukocyte activation at sites of inflammation, components of the NADPH oxidase complex become phosphorylated and assemble at the plasma membrane. The activated complex subsequently catalyzes the rapid reduction of molecular oxygen into superoxide ($O_2^{\bullet-}$) during a respiratory burst (*21*). Reduced oxygen species formed by leukocytes, such as $O_2^{\bullet-}$ and its dismutation product, hydrogen peroxide (H_2O_2), may then be converted into more reactive oxidizing species with potential for promoting DNA oxidation during chronic inflammation. In support of this hypothesis, in vitro studies demonstrate that stimulated leukocytes are capable of inducing genotoxic effects, such as DNA strand breaks (*22*, *23*), sister chromatid exchanges (*24*), mutation (*25*−*27*), and promotion of neoplastic transformation in nearby cells (*25*, *28*). Halogenating oxidants have

† This work was supported by a grant from the Department of Defense (BC980289) and by grants from the National Institutes of Health (HL62526 and HL61878 to S.L.H. and HL58758 to J.Q.). Z.S. is a recipient of a Medical Research Fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

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10.1021/bi001961t CCC: $20.00
Published on Web 01/26/2001

also been shown to be mutagenic to bacteria (*29*, *30*). A role in cancer development has also been suggested for the leukocyte peroxidase myeloperoxidase (MPO)[1] based upon its ability to convert procarcinogens into genotoxic intermediates and the modification of certain xenobiotics into more potent carcinogens (*31*−*34*). Recent studies also suggest a genetic link between MPO and the risk for developing cancer. The incidence of a specific polymorphism in the promotor region of the MPO gene has been demonstrated to be in genetic disequilibrium with the incidence of several cancers (*35*, *36*).

The precise chemical mechanisms linking inflammation, leukocyte activation, and DNA oxidative damage in vivo have not yet been fully characterized. Most studies have focused on the ability of free transition metal ions, like Fe^{2+} or Cu^{2+}, to catalyze conversion of leukocyte-generated reduced oxygen species (i.e., $O_2^{\bullet-}$ and H_2O_2) into the more reactive hydroxyl radical ($^{\bullet}OH$) through classic Haber-Weiss and Fenton chemistries (eq 1) (*37*−*39*).

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{M^{2+}} {}^{\bullet}OH + OH^- + O_2 \quad (1)$$

Eosinophils and neutrophils are endowed with distinct peroxidases, MPO and eosinophil peroxidase (EPO), respectively, which can utilize halides (X^-) as substrates to form hypohalous acids (HOX) (eq 2) (*13*, *40*).

$$X^- + H_2O_2 + H^+ \rightarrow HOX + H_2O \ (X = Cl \text{ or } Br) \quad (2)$$

These potent cytotoxic oxidants are microbicidal and are presumed to play a key role in the normal functions of leukocytes during innate host defenses (*41*−*43*). Recently, activated eosinophils and neutrophils (as well as their corresponding isolated peroxidases, EPO and MPO, respectively) were shown to promote oxidative damage of DNA, RNA, and the nucleotide pool of target cells through halide-dependent formation of $^{\bullet}OH$ (eq 3) (*44*).

$$O_2^{\bullet-} + HOX \rightarrow {}^{\bullet}OH + X^- + O_2 \ (X = Cl \text{ or } Br) \quad (3)$$

This reaction is analogous to the Haber-Weiss reaction (eq 1) where H_2O_2 is replaced with HOCl or HOBr. In the absence of metal ions, eq 3 is at least 6 orders of magnitude faster than the Haber-Weiss reaction (*45*−*47*).

Although the contribution of oxidative processes to carcinogenesis is now widely accepted, the precise chemical pathways involved remain unclear. A powerful method for unraveling the mechanisms through which DNA damage occurs is through the detection of distinct stable markers of free radical reactions. For example, markers of DNA damage by $^{\bullet}OH$, reactive nitrogen species, reactive chlorinating species, and aldehydes have been used to implicate specific chemical mechanisms of DNA damage in vivo (*48*−*55*). Although eosinophil recruitment is characteristic of many cancers (*56*−*58*) and numerous chronic parasitic infections are associated with an increased risk for development of cancer (*7*−*10*, *59*), no stable DNA oxidation products specific for eosinophils have been described.

Nearly two decades ago, the unique ability of eosinophils to generate brominating oxidants via the EPO/H_2O_2/Br^- system was first reported (*60*). Recently, the sensitivity and specificity of mass spectrometry has permitted the direct demonstration that brominating oxidants are a distinct class of oxidants formed by eosinophils in vivo (*61*). Since EPO is the only mammalian enzyme known to selectively generate reactive brominating species (*60*) at physiological concentrations of halides (100 mM Cl^-, 20−150 μM Br^-, and 0.1−0.6 μM I^- in plasma; *61*, and references therein), brominated products have the potential to serve as molecular markers that identify sites of EPO-mediated oxidative damage. In this study, we examined the ability of brominating oxidants and isolated EPO to promote oxidation of nucleosides and double-stranded DNA. Multiple distinct brominated products of nucleosides and DNA were observed using a combination of various mass spectrometric and NMR methods. The chemical reactions that are involved are characterized, and preferred targets for modification in double-stranded DNA are defined. A rationale for selective modification of adenine, but not guanine, in double-stranded DNA is also presented, based upon stereochemical criteria. The specific brominated nucleoside adducts that are identified may thus serve as molecular markers that permit identification of sites where brominating oxidants contribute to DNA damage.

EXPERIMENTAL PROCEDURES

Materials

Organic solvents (HPLC grade), H_2O_2 (30%; ACS grade), H_3PO_4, NaH_2PO_4, and Na_2HPO_4 were obtained from Fisher Chemical Co. (Pittsburgh, PA). Chelex-100 resin (200−400 mesh, sodium form) was obtained from Bio-Rad (Hercules, CA). Methanesulfonic acid and bromine were purchased from Fluka Chemical Co. (Ronkonkoma, NY). Heavy isotope-labeled adenosine (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) was obtained from Cambridge Isotope Laboratories (Andover, MA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Methods

General Procedures. Porcine EPO was isolated using the method of Jorg (*62*). Peroxidase activity during purification was monitored by the guaiacol oxidation assay (*41*). Purity of EPO preparations was assured before use by demonstrating an RZ of >0.95 (A_{415}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm the absence of contaminating MPO activity (*63*). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described previously (*64*). Trace levels of contaminating EPO were then removed by cation exchange chromatography (*65*). The purity of isolated MPO was established by demonstrating an RZ of >0.85 (A_{430}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm the absence of contaminating EPO activity (*63*). Enzyme concentrations were determined spec-

[1] Abbreviations: 8-BrA, 8-bromoadenine; 8-BrdA, 8-bromo-2′-deoxyadenosine; Br-dC, bromo-2′-deoxycytidine; 8-BrG, 8-bromoguanine; 8-BrdG, 8-bromo-2′-deoxyguanosine; Br-dT, bromo-2′-deoxythymidine; EPO, eosinophil peroxidase; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MS, mass spectrometry; *m*/*z*, mass-to-charge ratio; $[M + H]^+$, molecular ion; MPO, myeloperoxidase; MRM, multiple-reaction monitoring; SIM, selected ion monitoring; TFA, trifluoroacetic acid; UV, ultraviolet−visible.

trophotometrically utilizing extinction coefficients of 89 000 and 112 000 M^{-1} cm^{-1}/heme for MPO (*66*) and EPO (*67*, *68*), respectively. The concentration of the MPO dimer was calculated as half the indicated concentration of the heme-like chromophore.

Buffers were prepared with Chelex-treated distilled deionized water. HOBr, free of Br^- and bromate, was prepared the day of use from liquid bromine as described previously (*69*). HOBr was quantified spectrophotometrically (ϵ_{331} = 315 M^{-1} cm^{-1}) as its conjugate base, OBr^- (*70*). Fresh stock solutions of *N*-bromotaurine or *N*,*N*-dibromotaurine were prepared in phosphate buffer (20 mM, pH 7.0) by incubating the HOBr/OBr^- mixture with either a 100-fold molar excess of taurine or 0.5 molar equiv of taurine, respectively, immediately prior to use. The concentrations of H_2O_2 (ϵ_{240} = 39.4 M^{-1} cm^{-1}) (*71*), *N*-bromoamine (ϵ_{288} = 430 M^{-1} cm^{-1}) (*72*), and *N*,*N*-dibromoamine (ϵ_{336} = 371 M^{-1} cm^{-1}) (*72*) were determined spectrophotometrically. Production of H_2O_2 by the glucose/glucose oxidase system was assessed by oxidation of Fe(II) and formation of an Fe(III)−thiocyanate complex (*73*). ^{13}C- and ^{15}N-labeled bromoadenine (Br[$^{13}C_5$,$^{15}N_5$]A) was synthesized for use as an internal standard by the reaction of ^{13}C- and ^{15}N-labeled adenosine with an equimolar amount of HOBr in 50 mM phosphoric acid, and the products were identified and quantified by HPLC with on-line UV (254 nm) spectroscopy and electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS).

Bromination of Free Deoxyribonucleosides and Calf Thymus DNA. The DNA bases 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and 2′-deoxythymidine (dA, dC, dG, and dT, respectively) (10 mM) or Chelex-treated calf thymus DNA (1 mg/mL) was brominated by addition of oxidant (HOBr, *N*-monobromoamine, *N*,*N*-dibromoamine, H_2O_2, or glucose to the glucose/glucose oxidase system) in sodium phosphate buffer (20 mM, pH 7.0, unless otherwise indicated) at 37 °C for either 60 min or overnight under the conditions described in the figure legends. Reactions were stopped by addition of methionine (10 mM). Experiments utilizing the glucose/glucose oxidase system for H_2O_2 generation were performed in the presence of 100 μg/mL glucose and either 20 (0.005 unit/mL) or 100 ng/mL (0.025 unit/mL) glucose oxidase (grade II, Boehringer Mannheim, Indianapolis, IN) at 37 °C overnight. Preliminary studies demonstrated that under these conditions, constant fluxes of H_2O_2 at approximately 10 and 50 μM/h, respectively, are formed.

Sample Preparation. Following oxidation, DNA was pelleted at 0 °C by adding 100% ethanol and then washed with 70% ethanol. Samples were then resuspended in 500 μL of H_2O, and the synthetic ring-labeled internal standard (Br[$^{13}C_5$,$^{15}N_5$]A) was added. DNA was then hydrolyzed with methanesulfonic acid (100 μM) at 90 °C for 2 h in gastight vials evacuated of air and under a blanket of argon. Hydrolysates were resuspended in 500 μL of Chelex-treated H_2O. The adenine content was then quantified by reverse phase HPLC with UV detection (254 nm). Prior to LC/ESI/MS/MS analysis, natural and heavy isotope-labeled 8-bromoadenine (8-BrA) and 8-bromoguanine (8-BrG) were partially purified from the DNA hydrolysates by passage over a Supelclean LC-C_{18} SPE minicolumn (Supelclean LC-18 SPE tubes, 3 mL; Supelco Inc., Bellefonte, PA). Briefly, the hydrolysates were loaded onto minicolumns which had been pre-equilibrated with 0.1% trifluoroacetic acid (TFA). Salts and the majority of DNA bases were eluted from the column through sequential washes (2 mL) of 0.1% TFA. Brominated bases were then eluted with 2 mL of 20% methanol in 0.1% (v/v) trifluoroacetic acid (TFA in H_2O). Fractions were dried, reconstituted with H_2O, and subjected to LC/ESI/MS/MS analysis.

Reverse Phase HPLC Analysis of Modified DNA Bases. Analysis of free deoxyribonucleosides was performed on a Beckman Gold HPLC system equipped with a photodiode array detector. Separations were performed on a C18 column (Beckman Ultrasphere, 5 μm, 4.6 mm × 250 mm) equilibrated with solvent A [0.1% TFA (pH 2.5)]. The products were eluted at a flow rate of 1 mL/min with a linear gradient generated with solvent B [0.1% TFA in methanol (pH 2.5)] as follows: 0% solvent B for 5 min, from 0 to 100% solvent B over the course of 30 min, and 100% solvent B for 10 min. Products were monitored on a diode array detector and quantified at 254 nm employing standard curves constructed with authentic synthetic standards.

Mass Spectrometry. Mass spectrometric analyses were performed on a Quattro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, U.K.) equipped with an electrospray ionization (ESI) probe and interfaced with an HP 1100 HPLC system (Hewlett-Packard, Wilmington, DE) with a photodiode array detector. DNA bases were resolved on an Ultrasphere C18 column (Beckman, 5 μm, 4.6 mm × 250 mm) at a flow rate of 1 mL/min and a linear gradient between H_2O (with 0.3% formic acid) and methanol (with 0.3% formic acid) over the course of 30 min. The column eluent was split (970 μL/min to the UV detector and 30 μL/min to the mass detector) and analyzed by the mass spectrometer in the positive ion mode with a cone potential of 50 eV. Analytes were detected using the total ion scan mode or the selected ion monitoring (SIM) mode, or with multiple-reaction monitoring (MRM) as indicated in the text and legends.

Quantification of 8-BrA was performed following resolution on a Luna C18 column (Phenomenex, 5 μm, 1.0 mm × 30 mm) at a flow rate of 25 μL/min and an isocratic condition of 30% CH_3CN in H_2O with 0.05% formic acid and 0.005% TFA. 8-BrA and its corresponding heavy isotope-labeled internal standard, 8-Br[$^{13}C_5$,$^{15}N_5$]A, were detected using positive ion electrospray ionization mass spectrometry in the MRM mode. The transitions that were monitored were those between the molecular cation of the ^{79}Br isotopomer and the characteristic brominated daughter ion formed from loss of NH_3 [i.e., mass-to-charge ratio (*m*/*z*) 214 → 197 for BrA and 224 → 206 for 8-Br[$^{13}C_5$,$^{15}N_5$]A].

NMR Studies. NMR samples were prepared by dissolving deoxyribonucleic acids and HPLC-purified brominated adducts in 100% D_2O. Analyses were performed at 25 °C with a Varian Inova 500 MHz NMR spectrometer (499.843 MHz for ^1H) equipped with a triple-resonance probe head and a shielded *Z*-gradient unit. ^1H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 in D_2O. For one-dimensional NMR and two-dimensional NOESY (mixing time of 400 ms) experiments, the intense HOD signal was attenuated by transmitter preirradiation.

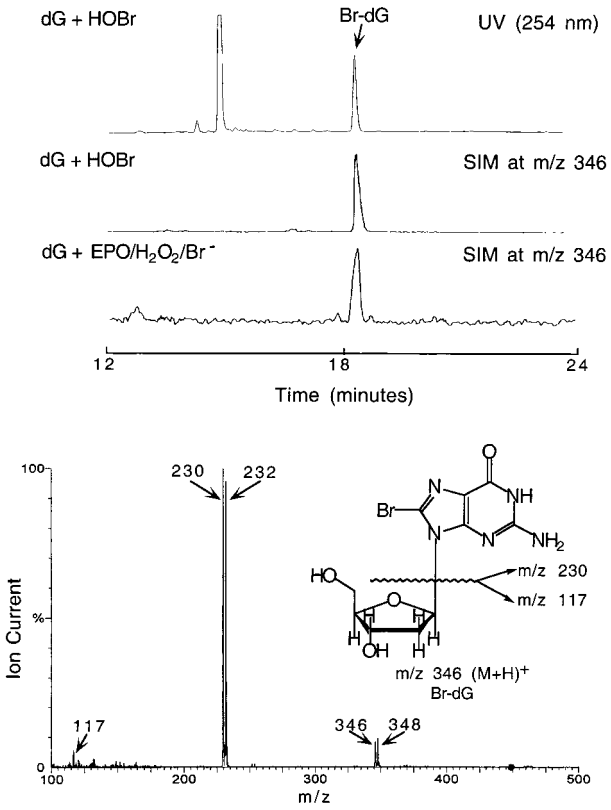

FIGURE 1: Analysis of reaction products formed upon exposure of dG to either HOBr or the EPO/H_2O_2/Br^- system. dG (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m*/*z* 346, the anticipated *m*/*z* of a protonated monobrominated adduct of dG. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dG, structure and fragmentation pattern shown in the inset) is also shown.

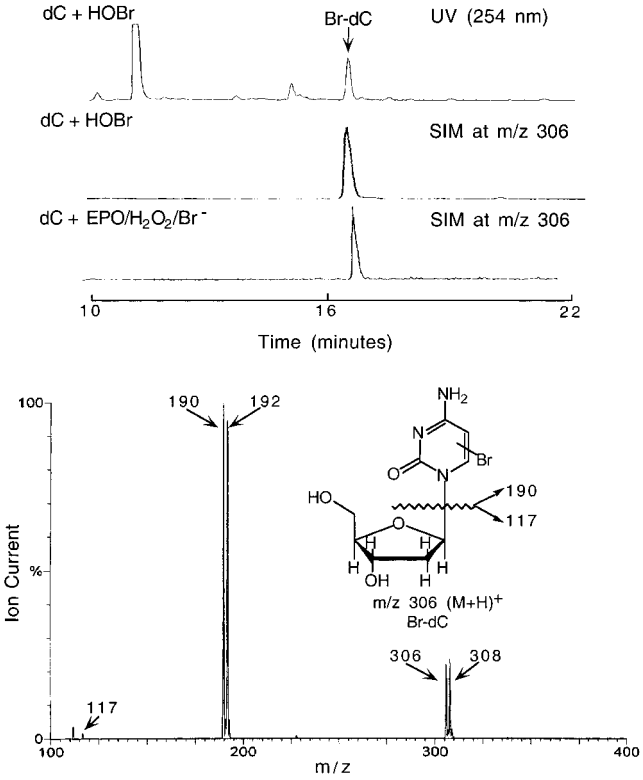

FIGURE 2: Analysis of reaction products formed upon exposure of dC to either HOBr or the EPO/H_2O_2/Br^- system. dC (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m*/*z* 306, the anticipated *m*/*z* of a protonated monobrominated adduct of dC. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dC, structure and fragmentation pattern shown in the inset) is also shown.

RESULTS

Free Nucleosides Are Targets for Oxidation by Eosinophil Peroxidase at Plasma Levels of Halides. In initial experiments, each of the individual 2′-deoxynucleosides (dG, dC, dA, or dT) was incubated with isolated EPO, H_2O_2, and plasma levels of Br^- (100 μM) and Cl^- (100 mM), and then the reaction products were analyzed by reverse phase HPLC with on-line UV detection (254 nm) and electrospray ionization mass spectrometry (ESI/MS) in the positive ion mode (Figures 1−4). Analysis of the oxidation products formed by exposure of dG to either HOBr or the EPO/H_2O_2/Br^- system in phosphate buffer at neutral pH demonstrated the formation of a major new product with a distinct retention time (Figure 1). The positive ion mass spectrum of the oxidized base generated by the EPO system is shown in the bottom panel of Figure 1 and is consistent with a monobrominated derivative of dG possessing a molecular ion $[M + H]^+$ with a mass-to-charge ratio (*m*/*z*) of 346. The mass spectrum also demonstrates the isotopic cluster expected for a monobrominated compound (1:1, M:M + 2), with ions of near equal relative abundance at *m*/*z* 346 ($[M + H]^+$ of the ^{79}Br-containing isotopomer) and *m*/*z* 348 ($[M + H]^+$ of the ^{81}Br-containing isotopomer). The mass spectrum also revealed fragment ions consistent with cleavage of the sugar−purine bond, generating the anticipated daughter ions arising from the 2′-deoxyribose group (*m*/*z* 117) and a monobrominated guanine moiety (*m*/*z* 230) (Figure 1, bottom panel and inset). Finally, analysis of the oxidation products formed by either reagent HOBr or the EPO/H_2O_2/Br^- system using LC/ESI/MS with monitoring in the selected ion monitoring mode (SIM) at *m*/*z* 346 $[M + H]^+$ demonstrated a single major product with an identical retention time (Figure 1). These results demonstrate that a stable ring-brominated adduct was formed following exposure of free dG to EPO-generated HOBr (Figure 1).

Similar results were obtained when dC was incubated with either reagent HOBr or isolated EPO in the presence of H_2O_2 and plasma levels of halides (Figure 2). A single major new product with a distinct retention time was observed during analysis by reverse phase HPLC with on-line UV detection (254 nm) and LC/ESI/MS analysis monitoring at *m*/*z* 306, the anticipated $[M + H]^+$ for the monobrominated species (Figure 2). Again, the full-scale positive ion ESI/MS spectrum of the major oxidation product was consistent with the formation of a monobrominated base, as illustrated in Figure 2.

When dA was incubated with HOBr in phosphate buffer at pH 7.0, no detectable products were apparent during HPLC

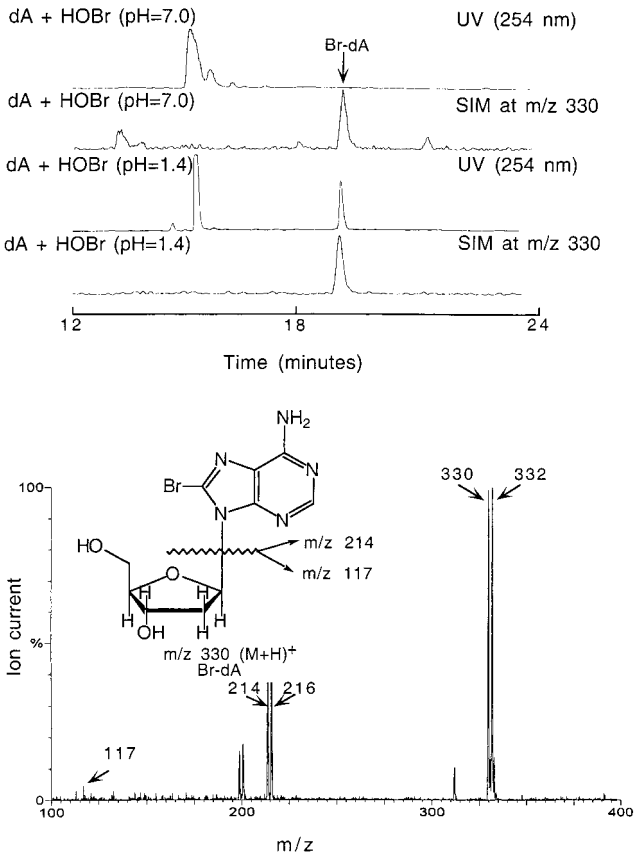

FIGURE 3: Analysis of reaction products formed upon exposure of dA to either HOBr or the EPO/H_2O_2/Br^- system. dA (10 mM) was incubated with HOBr (5 mM) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0 or 1.4) supplemented with NaCl (100 mM). In parallel, dA (10 mM) was incubated with the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m*/*z* 330, the anticipated *m*/*z* of a protonated monobrominated adduct of dA, as described in Experimental Procedures. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dA, structure and fragmentation pattern shown in the inset) is also shown.

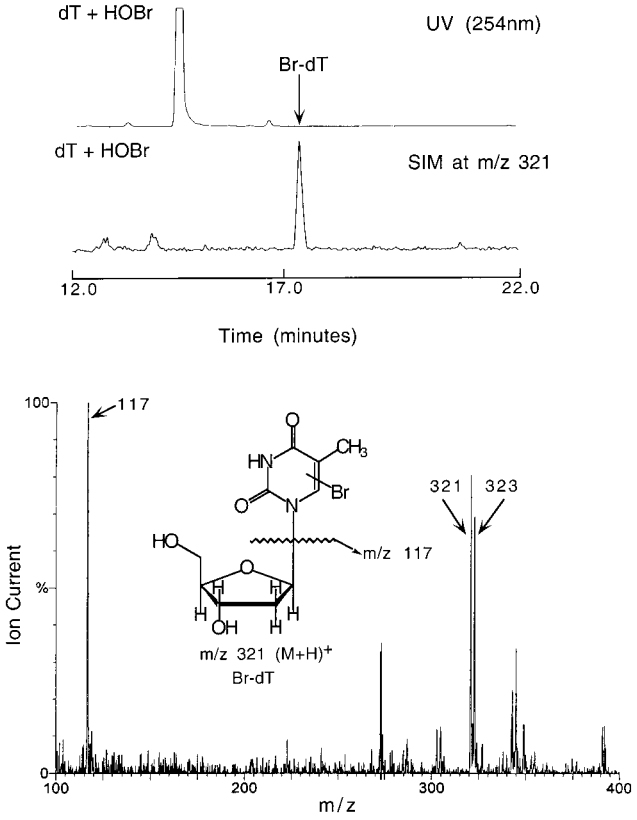

FIGURE 4: Analysis of reaction products formed upon exposure of dT to either HOBr or the EPO/H_2O_2/Br^- system. dT (10 mM) was incubated with either HOBr (5 mM) or the EPO/H_2O_2/Br^- system (57 nM EPO, 100 μM H_2O_2, and 100 μM NaBr) for 1 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with NaCl (100 mM). Products were subsequently analyzed by reverse phase HPLC with an on-line diode array detector (254 nm trace shown) and positive ion ESI-MS analysis using the single-ion monitoring (SIM) mode at *m*/*z* 321, the anticipated *m*/*z* of a protonated monobrominated adduct of dT. The full scan positive ion ESI mass spectrum of the major product generated by EPO (Br-dT, structure and fragmentation pattern shown in the inset) is also shown.

analysis by UV using a photodiode array detector (data for 254 nm shown); however, a product with the appropriate *m*/*z* of a monobrominated dA derivative was readily detected by LC/ESI/MS during SIM at *m*/*z* 330, the anticipated *m*/*z* for a monobrominated adduct. When dA was exposed to HOBr under acidic conditions, significant levels of Br-dA were apparent during HPLC analysis by both UV and ESI/MS detection (Figure 3). The molecular ion and fragmentation pattern of the product was again consistent with formation of the monobrominated species (Figure 3, bottom panel). Finally, exposure of dT to the major oxidant of the EPO/H_2O_2/Br^- system, HOBr, at neutral pH produced low levels of monobrominated dT (Br-dT) which was only detected by LC/ESI/MS in SIM at *m*/*z* 321 (anticipated *m*/*z* for the monobrominated adduct) (Figure 4). At low pH, Br-dT was formed in high yield, along with numerous other mono- and dibrominated degradation products. Collectively, these results demonstrate that all four free deoxyribonucleosides can serve as targets for bromination. However, the yields of Br-dG and Br-dC were substantially higher at neutral pH than those of Br-dA and Br-dT, suggesting that they will serve as primary targets among the free nucleobases.

Structural Identification of Brominated Purine Bases. Preliminary studies suggested that the monobrominated pyrimidine products that are formed are acid labile. Since our long-term interest is to identify stable brominated nucleotide adducts which might serve as in vivo markers for the detection of DNA damage by EPO-generated brominating oxidants, we focused our subsequent studies on structurally characterizing the monosubstituted brominated adducts of purines formed following exposure of bases to EPO-generated HOBr. To identify the sites on the heterocyclic rings where bromine was covalently attached, we initially utilized ^{1}H NMR methods. The one-dimensional spectra of Br-dG and the parent base, dG, are shown in Figure 5. Inspection of the spectra reveals that the H_8 proton is lost in the brominated adduct, confirming the ring assignment of the monobrominated species as 8-Br-2′-dG (also known as Br-dG). Analysis of the proton NMR spectra (one-dimensional) of monobrominated and native dA did not permit unambiguous assignment of the location for ring bromination. Subsequent two-dimensional NOESY analyses

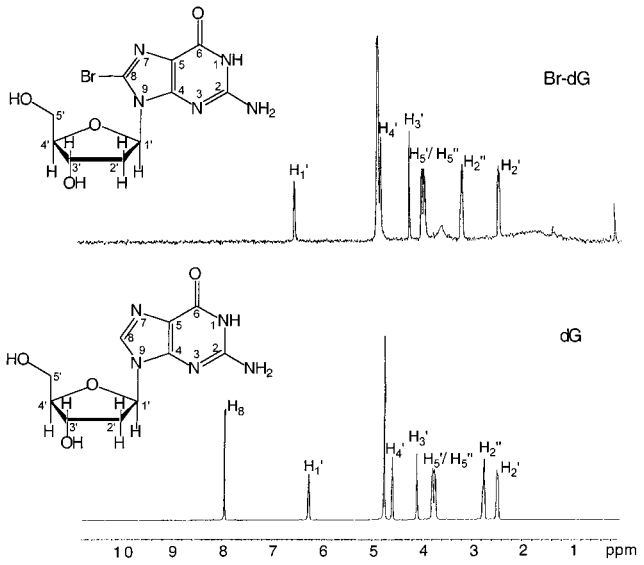

FIGURE 5: ^{1}H NMR spectra of BrdG and dG. The monobrominated oxidation product of dG formed following exposure to the EPO/H_2O_2/Br^- system (*m*/*z* 346) was isolated by reverse phase HPLC, dried, and then analyzed by ^{1}H NMR in D_2O as described in Experimental Procedures. For comparison, the ^{1}H NMR spectrum of dG is shown. Note the disappearance of the resonance derived from the H_8 proton in the ^{1}H NMR spectrum of BrdG, compared to that of dG.

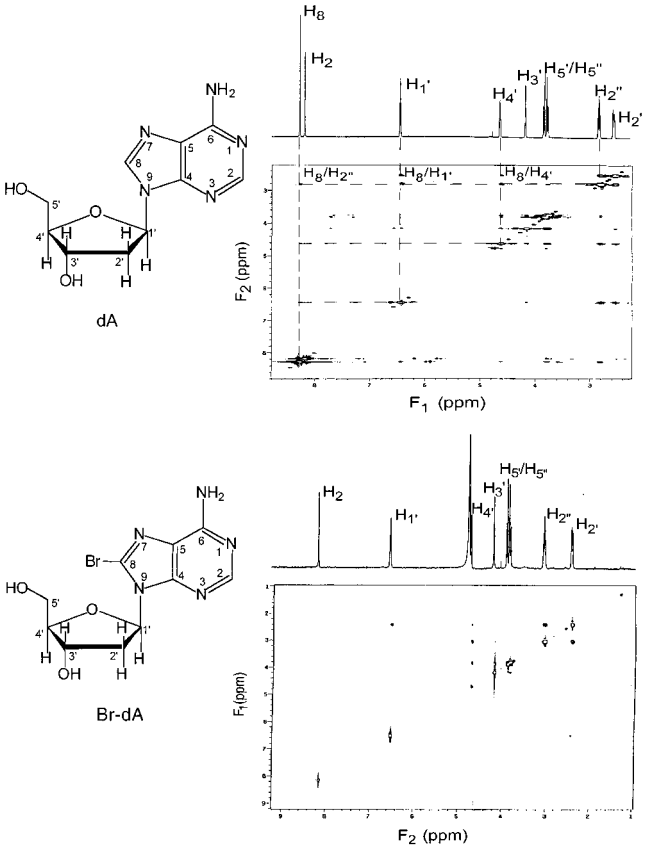

FIGURE 6: Two-dimensional NOESY spectra of dA and Br-dA. The monobrominated oxidation product of dA formed following exposure to the EPO/H_2O_2/Br^- system (*m*/*z* 330) was isolated by reverse phase HPLC. Both dA and the monobrominated product were each then analyzed by two-dimensional NMR as described in the text. Note that the two-dimensional NOESY spectrum of dA shows the NOE cross-peak between H_8 and $H_{2'}$ which are spatially close. In contrast, the two-dimensional NOESY spectrum of Br-dA shows the disappearance of H_8 and its NOE connection with $H_{2'}$.

demonstrated that the aromatic H_8 proton of dA exhibits several NOE cross-peaks to the adjacent sugar ring protons (Figure 6, top panel), while these NOE connections disappear in Br-dA (Figure 6, bottom panel). Thus, the H_8 position of dA is replaced with bromine, and the monobrominated dA species that forms is 8-Br-2′-dA (also known as Br-dA).

Characterization of Reaction Requirements and Reactive Intermediates Involved in Br-dG Formation by EPO. We next examined the reaction requirements for Br-dG formation by isolated EPO. Following incubation of dG with EPO, a H_2O_2-generating system (glucose and glucose oxidase; ~10 μM H_2O_2/h under the conditions that were employed), and plasma levels of halides, Br-dG was formed (Figure 7, top panel, complete system). The time course for EPO-dependent bromination of dG under these conditions is illustrated in Figure 8. Near-maximal Br-dG formation was observed within 2 h. If 1 mol of H_2O_2 is required to generate 1 mol of brominating equivalent from EPO, and 1 mol of brominating equivalent is used to generate Br-dG, then the overall yield of EPO-dependent bromination of dG was ~6.5% under the conditions employed here. Formation of Br-dG required the presence of each component of the EPO/H_2O_2/Br^- system. Moreover, addition of either heme inhibitors, like azide, or scavengers of reactive halogenating species, like the thioether methionine, completely ablated formation of the brominated base (Figure 7, top panel). In contrast, BrdG formation still was observed in reaction mixtures containing N_α-acetyllysine, a low-molecular weight surrogate for protein amino groups. These results suggested that *N*-bromoamines might promote nucleotide bromination reactions. Exposure of excess dG to an equivalent amount of distinct brominating intermediates, including HOBr and OBr^-, *N*-monobromotaurine, *N*,*N*-dibromotaurine, and HOBr with Br^- (a Br_2-containing system), revealed that *N*-bromoamines were the preferred brominating intermediates (~40% overall yield, per mole of brominating equivalent) (Figure 7, bottom panel). Finally, the bromide concentration dependence of Br-dG formation by the EPO/H_2O_2 system in medium containing 100 mM Cl^- was examined and is illustrated in Figure 9. Even in the face of a >1000-fold molar excess of Cl^-, EPO effectively generates Br-dG across the physiological concentration range of Br^- (20−150 μM).

8-Bromoadenine Is the Major Stable Purine Oxidation Product Formed following Exposure of DNA to the HOBr/OBr^- System. A critical question was whether bases in double-stranded DNA would serve as a target for bromination. To test this, we exposed calf thymus DNA to low levels of reagent HOBr and then DNA was recovered, washed, hydrolyzed, and analyzed by LC/ESI/MS. Interestingly, BrA, but not BrG, was detected in the SIM mode while monitoring at *m*/*z* 214 and 230, the *m*/*z* anticipated for the molecular ions of BrA and BrG, respectively (Figure 10). The identity of the ion at *m*/*z* 214 as BrA was confirmed by demonstration of the retention time being identical to that of authentic BrA (not shown) and analysis of the full scan positive ion mass spectrum of the analyte, which revealed the characteristic isotopic cluster of the monobrominated adduct (Figure 10, inset).

Demonstration That the EPO/H_2O_2/Br^- System Generates BrA in Double-Helix DNA. To develop a more sensitive and specific method for the identification of BrA in DNA, as

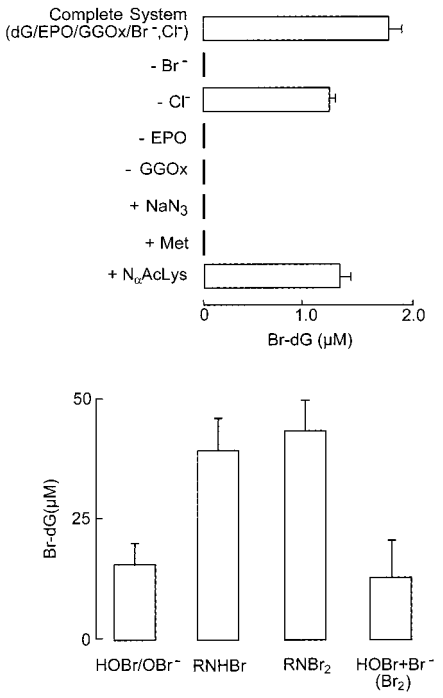

FIGURE 7: Reaction characteristics of 8-BrdG formation by eosinophil peroxidase. (Top) dG (2 mM) was incubated with eosinophil peroxidase (57 nM) and a H_2O_2-generating system (10 μM/h; 100 μg/mL glucose and 20 ng/mL glucose oxidase) in sodium phosphate buffer (20 mM, pH 7.0) supplemented with plasma levels of halides (100 mM NaCl and 100 μM NaBr) at 37 °C (Complete System). Where indicated, components of the Complete System were omitted, or the indicated additions were included in the reaction mixtures: sodium azide (1 mM), methionine (10 mM), or N_α-acetyllysine (1 mM). Reaction products were then analyzed by reverse phase HPLC at 254 nm, and the content of BrdG that formed was determined as described in Experimental Procedures. Data represent the means ± standard deviation (SD) of three independent experiments. (Bottom) dG (2 mM) was incubated with 10 μM (final concentration) HOBr/OBr^-, *N*-bromotaurine (RNHBr), *N*,*N*-dibromotaurine ($RNBR_2$), or HOBr/OBr^- with 100 mM NaBr in reaction buffer [20 mM sodium phosphate (pH 7.0)] at 37 °C for 1 h. Reactions were quenched by addition of methionine (10 mM), and the production of BrdG was assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of three independent experiments.

might be required for detection of the modified base in vivo, we sought to develop a tandem mass spectrometry (MS/MS)-based method for its detection. MS/MS analysis of each isotopomer of BrA produced by EPO-generated HOBr was performed, and the spectra and proposed fragmentation patterns are illustrated in Figure 11. The proposed fragmentation pattern for the monosubstituted adenine is similar to that reported for authentic adenine (*74*). The parent ion (*m*/*z* 214/216), by the loss of NH_3, forms a major characteristic brominated daughter ion at *m*/*z* 197/199 with a moderate collision energy of ~30 eV (Figure 11).

In a final series of experiments, we exposed calf thymus DNA to a low flux of H_2O_2 (10 μM/h) and plasma levels of halides in the presence and absence of EPO and utilized stable isotope dilution LC/ESI/MS/MS analysis to determine whether Br-A was formed. Typical chromatograms of DNA hydrolysates analyzed by HPLC with on-line tandem mass spectrometry in the positive ion mode while monitoring the transitions of *m*/*z* 214 → 197 for BrA and *m*/*z* 224 → 206 for the Br[$^{13}C_5$,$^{15}N_5$]A internal standard are shown in Figure

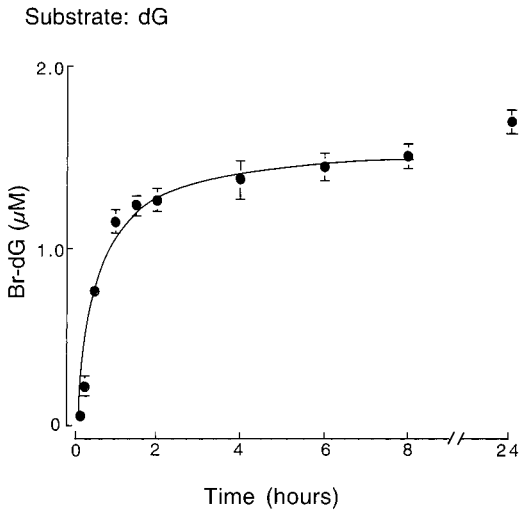

FIGURE 8: Time course study of bromo-dG formation by eosinophil peroxidase. dG (2 mM) was incubated with eosinophil peroxidase (57 nM), NaCl (100 mM), NaBr (100 μM), glucose (100 μg/mL), and glucose oxidase (20 ng/mL) in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C. At the indicated times, excess methionine (10 mM) was added and the production of BrdG assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of triplicate determinations for a representative experiment performed at least three independent times.

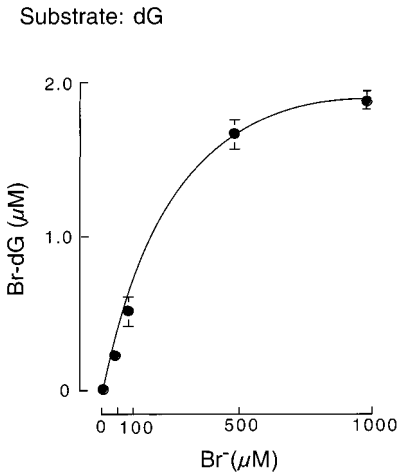

FIGURE 9: Bromide dependence of dG bromination by eosinophil peroxidase. dG (2 mM) was incubated with eosinophil peroxidase (57 nM), NaCl (100 mM), glucose (100 μg/mL), glucose oxidase (20 ng/mL), and the indicated concentrations of sodium bromide in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C. Reactions were quenched by addition of excess methionine (10 mM), and the production of BrdG was assessed by reverse phase HPLC at 254 nm as described in Experimental Procedures. Data represent the means ± SD of triplicate determinations for a representative experiment performed at least three independent times.

12. A significant level of BrA was formed only in the presence of EPO (Figure 12).

DISCUSSION

The contribution of oxidative processes to carcinogenesis is now widely accepted (*1*−*10*). Much progress in this area involves use of stable markers of free radical reactions to identify specific chemical mechanisms of DNA damage in vivo. For example, hydroxylated, nitrated, aldehyde-modified, and chlorinated bases have been characterized and used to determine mechanisms of DNA, RNA, and nucleotide

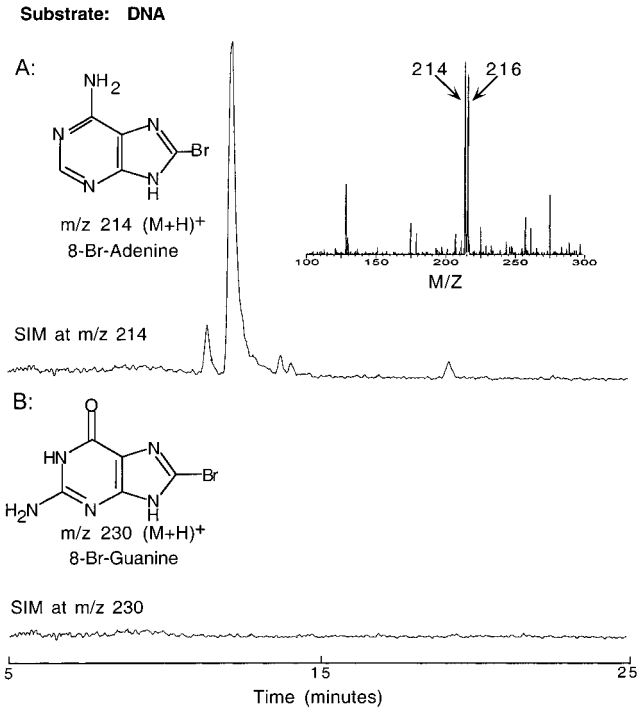

FIGURE 10: 8-Bromoadenine, but not 8-bromoguanine, is formed following exposure of DNA to the EPO-generated oxidant, HOBr. Calf thymus DNA (1 mg/mL) was incubated with HOBr (100 μM) in sodium phosphate buffer (20 mM, pH 7.0) for 60 min. The reaction was quenched by addition of excess methionine (10 mM), and then DNA was isolated, hydrolyzed, and analyzed by reverse phase HPLC with on-line ESI-MS analysis as described in Experimental Procedures. Production of BrA and BrG was assessed by analysis in the selected ion monitoring (SIM) mode at *m*/*z* 214 and 230, respectively. A major ion with a retention time identical to that of authentic 8-BrA, but not to that of 8-BrG, was observed. (Inset) In a separate HPLC run, the full scale mass spectrum of the ion eluting with *m*/*z* 214 and the retention time of 8-BrA was obtained. The identity of the DNA oxidation product (8-BrA) was further confirmed by noting the isotopic cluster expected for a monobrominated adenine adduct, with ions with a 1:1 M:M + 2 relative intensity at *m*/*z* 214 and 216.

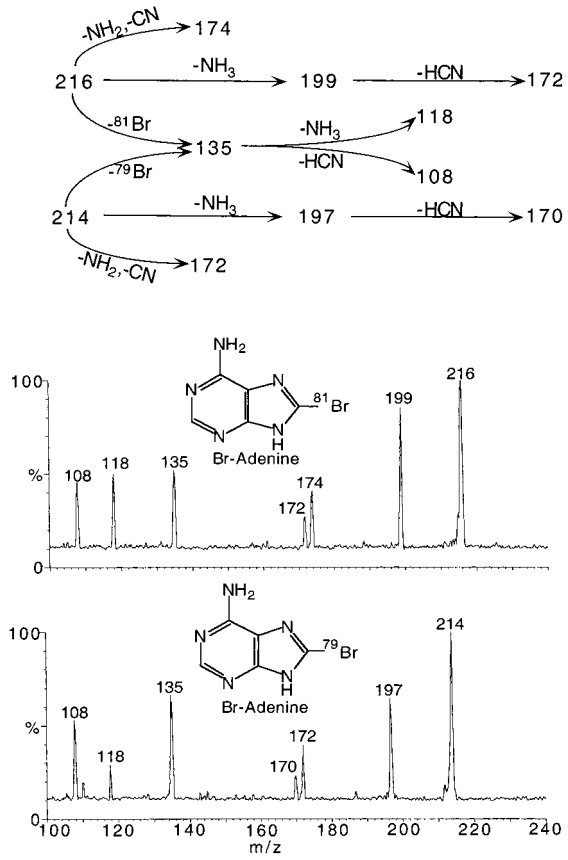

FIGURE 11: Positive ion electrospray ionization tandem mass spectrometric analysis of 8-bromoadenine. 8-BrG generated during HOBr-mediated oxidative modification of DNA (Figure 10) was analyzed by reverse phase HPLC with on-line electrospray ionization tandem mass spectrometry, as described in Experimental Procedures. (Top) Scheme for the proposed fragmentation patterns of protonated forms of ^{79}Br and ^{81}Br isotopomers of 8-bromoadeninine (*m*/*z* 214 and 216, respectively). (Middle) Daughter ion analysis of *m*/*z* 216 ([M + H]$^+$ for the ^{81}Br isotopomer) revealed the following ions: *m*/*z* 199 ([M + H]$^+$ − NH_3), 174 ([M + H]$^+$ − NH_2 − CN), 172 ([M + H]$^+$ − NH_3 − HCN), 135 ([M + H]$^+$ − ^{81}Br), 118 ([M + H]$^+$ − ^{81}Br − NH_3), and 108 ([M + H]$^+$ − ^{81}Br − HCN). (Bottom) Daughter ion analysis of *m*/*z* 214 ([M + H]$^+$ for the ^{79}Br isotopomer) revealed the following ions: *m*/*z* 197 ([M + H]$^+$ − NH_3), 172 ([M + H]$^+$ − NH_2 − CN), 170 ([M + H]$^+$ − NH_3 − HCN), 135 ([M + H]$^+$ − ^{79}Br), 118 ([M + H]$^+$ − ^{79}Br − NH_3), and 108 ([M + H]$^+$ − ^{79}Br − HCN).

damage in vitro and in vivo (*48*−*55*). However, the potential role of brominating oxidants in DNA damage and cancer development has not received much attention. The results of the studies presented here suggest that formation of reactive brominating species by the EPO/H_2O_2/Br^- system of eosinophils may be one pathway these cells contribute to oxidative modification of DNA and the nucleotide pool. Recent studies identify brominating oxidants as a distinct class of oxidants formed following eosinophil activation in vivo (*61*). Moreover, numerous cancers are notable for a significant eosinophilic infiltration in the cancerous tissues. The specific brominated bases that are identified may thus serve as markers for future studies aimed at determining the potential role of brominating oxidants in DNA oxidative damage in vivo.

On the basis of the results from the present report and recent published studies (*44*), we have generated a model of potential pathways through which brominating oxidants may contribute to oxidative modification of free bases, RNA, and DNA (Figure 13). Upon activation, the NADPH oxidase complex of eosinophils forms $O_2^{\bullet-}$, which both spontaneously and enzymatically dismutates to form H_2O_2. Concomitantly, eosinophil activation leads to the secretion of EPO into the extracellular compartment. In the presence of plasma levels of Br^-, EPO generates brominating oxidants such as HOBr, which can directly brominate free nucleosides and DNA forming stable monobrominated adducts (Figure 13). Identification of brominated adducts of each free base was confirmed by HPLC with on-line ESI-MS analysis. In the case of brominated purine bases, structural characterization as the 8-bromo-substituted analogues of adenine and guanine was further confirmed by NMR and tandem mass spectrometry. EPO-generated HOBr can also react with $O_2^{\bullet-}$ to form a $^{\bullet}$OH-like oxidant (eq 3) (Figure 13). The content of 8-hydroxyguanine, a marker of $^{\bullet}$OH-dependent DNA damage, was recently shown to significantly increase in DNA, RNA, and the nucleotide pool of cells exposed to a hypohalous acid generating system and enhanced intracellular $O_2^{\bullet-}$ (*44*).

One remarkable feature of these results is the finding that addition of a large molar excess of primary amines failed to

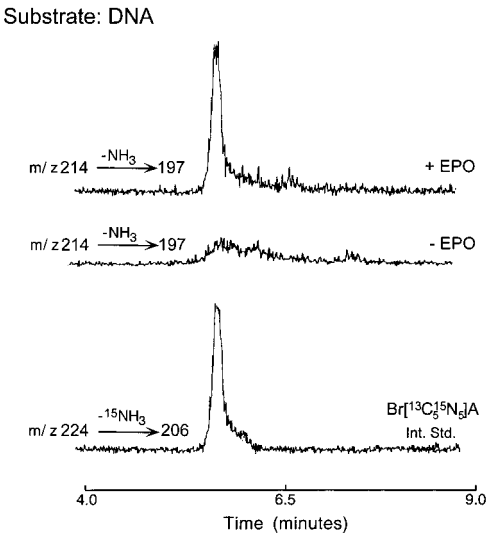

FIGURE 12: Positive ion electrospray ionization tandem mass spectrometric detection of 8-bromoadenine formed following DNA modification by the EPO/H_2O_2/Br^- system. Calf thymus DNA (1 mg/mL) was incubated overnight at 37 °C with a H_2O_2-generating system (10 μM/h; 100 μg/mL glucose and 20 ng/mL glucose oxidase) in Chelex-treated sodium phosphate buffer (20 mM, pH 7.0) supplemented with plasma levels of halides (100 mM NaCl and 100 μM NaBr) in either the presence (+EPO) or absence (−EPO) of EPO (105 nM). DNA was then isolated, heavy isotope-labeled internal standard (Br[$^{13}C_5$,$^{15}N_5$]A) added, and formation of 8-BrA assessed by HPLC with on-line tandem mass spectrometry using the multiple-reaction monitoring mode as described in Experimental Procedures. 8-BrA was detected while monitoring the transition between the molecular ion [*m*/*z* 214 ([M + H]$^+$ for the ^{79}Br isotopomer)] and the characteristic daughter ion at *m*/*z* 197 ([M + H]$^+$ − NH_3). The corresponding transition was monitored for the isotopically labeled internal standard.

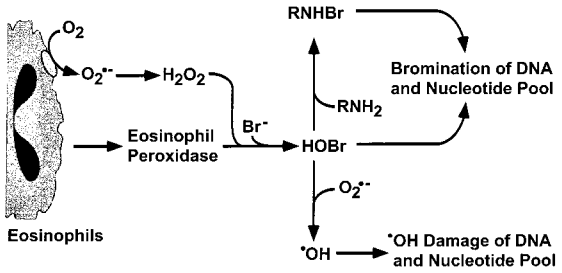

FIGURE 13: Model of potential pathways through which EPO-generated brominating oxidants may contribute to DNA oxidative damage.

block nucleoside bromination. These results are consistent with the finding that *N*-bromoamines serve as preferred brominating intermediates (Figure 7). Protein-bound and free primary amines are some of the most abundant nucleophilic moieties that reactive halogenating species will encounter in vivo. The ability of *N*-bromoamines to promote bromination of free nucleosides and adenine in DNA is thus anticipated to prolong the effective half-life of brominating agents formed in vivo and to help "funnel" brominating equivalents toward stable ring-brominated adducts of nucleosides (Figure 13). Similar results were observed for bromination of the aromatic amino acid tyrosine (*75*). In this respect, bromination reactions are distinct from results reported for the chlorination of the pyrimidine cytosine (*48*), the double bond of sterols (*76*), and phenolic targets such as tyrosine (*77*), all of which appear to be mediated by molecular chlorine, and not by *N*-chloroamines. Moreover,

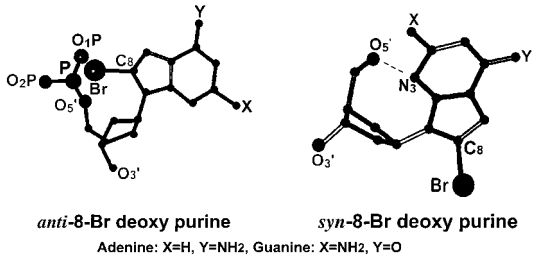

FIGURE 14: Structures of 8-bromopurines having anti and syn glycosyl torsions for the bases. 8-Bromopurines with the anti glycosyl conformation will result in short contacts between bromine and atoms of the sugar−phosphate backbone chain. The steric hindrance is avoided in 8-bromopurines having bases in the syn conformation, which is stabilized by an $O_{5'}$−H···N_3 hydrogen bond. The conditional demands for syn purine bases in double-helix DNA are not favorable for guanine, as opposed to adenine, as explained in the text.

whereas bromination of free nucleosides such as dG and dC occurs readily at neutral pH, an acidic environment is required for chlorination of all nucleosides (Z. Shen, W. Wu, and S. L. Hazen, unpublished observations; *48*). It has been suggested that the abundance of the amino acid taurine within phagocytes (∼20 mM) may serve to trap oxidized forms of halides and protect critical intracellular targets from oxidation and halogenation (*40*). The facile reduction of *N*-bromoamines resulting in nucleotide halogenation reactions at neutral pH raises the interesting possibility that high intracellular levels of the β-amino acid may instead promote modification of nucleobases in the free nucleotide pool, as well as accessible nucleotide-containing polymers such as various RNAs or DNAs.

Another interesting result in this study is the observation that 8-bromoadenine is a major stable purine oxidation product formed following exposure of double-stranded DNA to the EPO/H_2O_2/Br^- system, while no detectable 8-bromo adduct for guanine in DNA was formed. These results are the reverse of the rank order for bromination observed with free nucleosides at neutral pH. Conformational analysis of the steric requirements for incorporation of a halide into the C-8 position of purine bases both in free nucleosides and within the major groove of the DNA double helix provides a rationale for these observations (Figure 14). Addition of a bulky bromo group at the C-8 position ortho to the glycosyl link, due to steric constraints, forces the purine bases to adopt a syn glycosyl conformation (Figure 14) (*79*). In contrast, a smaller hydroxyl group at the C-8 position of purines, such as in formation of 8-hydroxyguanine or 8-hydroxyadenine, is readily accommodated in the anti glycosyl conformation. While a syn conformation for purine bases is quite frequent in free nucleosides, a change in the glycosyl torsion from anti to syn in double-stranded DNA would result in a significant free energy expenditure due to changes in the base pairing scheme (below). For purine bases either free or in the C2′-endo sugar pucker (B-DNA), a syn glycosyl conformation results in an additional stability contributed by an intramolecular O5′−H···N3 hydrogen bond (*79*). Therefore, bromination at C-8 in the free purine bases or the nucleosides can occur readily.

In contrast, as noted above, for bromination to occur at the C-8 position of either adenine or guanine in a DNA double helix, the purine base must convert its glycosyl conformation from anti to syn. In both cases, this conversion

will necessitate the transformation of a Watson−Crick base pair to a Hoogsteen pair (*80*). A Hoogsteen A·T base pair, like its Watson−Crick pair, has two hydrogen bonds and can be formed without loss of stability. For a G·C Hoogsteen pair to be formed with the G in the syn conformation, the complementary cytosine base needs to be protonated (*79*). This will impose a significant free energy restriction by the demand of a lower pK_a for such a transition to occur. In addition, in the Hoogsteen geometry a G·C pair loses stability by having only two hydrogen bonds, in contrast to three hydrogen bonds for its Watson−Crick geometry (*80*). These restrictions readily explain the predominance of 8-BrA formation and absence of any 8-BrG adducts, following exposure of calf thymus DNA to either reagent HOBr or the EPO/H_2O_2/Br^- system.

One critical question that has not yet been resolved is whether bromination of purine and pyrimidine targets takes place in vivo. Clearly, the probability of a brominating oxidant diffusing through a gauntlet of cytosolic scavengers unscathed before reaching a nuclear DNA base as its ultimate target will be a low-probability event (as are all DNA oxidation events). However, it should also be recognized that the oxidation event does not have to take place inside the cell nucleus, but may occur either within the cytosol (i.e., the nucleotide pool) or even within the extracellular compartment. Parasitic infections are accompanied by increased cell death and lysis at the site of inflammation. Moreover, brominated bases can be taken up and incorporated into DNA and RNA of cultured mammalian cells (*78*). Though mammalian cells are equipped with numerous surveillance mechanisms for removal of modified bases from the nucleotide pool, the fidelity of these systems is not absolute (*20*). Indeed, exposing cultured cells to brominated bases in the medium results in sister chromatid exchanges and mutation (*78*). We have recently demonstrated that bromination of extracellular targets (protein tyrosine residues) by activated eosinophils occurs in vivo at sites of inflammation (*61*). The ability of certain free nucleobases to undergo bromination at neutral pH suggests that similar events may occur in vivo. Thus, in the setting of a chronic parasitic infection where decades of eosinophil-mediated inflammatory injury can occur, bromination of extracellular or cytosolic nucleobases may occur.

In summary, the results presented here suggest that specific brominated DNA bases may serve as novel and specific markers for monitoring oxidative damage of DNA and the nucleotide pool by brominating oxidants. Moreover, they identify adenine, rather than guanine, as a more likely target for halogenation of purine bases within double-helix DNA. The detection of brominated bases in eosinophil-rich inflammatory lesions or cancers would strongly suggest that brominating oxidants formed by these cells contribute to the development of DNA damage in these disorders.

ACKNOWLEDGMENT

Mass spectrometry experiments were performed at the Mass Spectrometry Resource of the Cleveland Clinic Foundation and the Cleveland Mass Spectrometry Core Facility. NMR experiments were performed in the Cleveland Center for Structural Biology.

REFERENCES

1. Freeman, B. A., and Crapo, J. D. (1982) *Lab. Invest. 47*, 412−426.
2. Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M., and Harman, D. (1987) *Ann. Intern. Med. 107*, 526−545.
3. Demopoloulos, H., Pietronigro, D. D., and Seligman, M. L. (1983) *J. Am. Coll. Toxicol. 2*, 173−184.
4. Kaplan, R. P. (1987) *Adv. Dermatol. 2*, 19−46.
5. Gordon, L. I., and Weitzman, S. A. (1988) in *The Respiratory Burst and its Physiological Significance* (Sbarra, A. J., and Strauss, R. R., Eds.) pp 277−298, Plenum Press, New York.
6. Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Henderson, B. E. (1991) *Cancer Res. 50*, 7415−7421.
7. Rosin, M. P., Anwar, W. A., and Ward, A. J. (1994) *Cancer Res. 54*, 1929s−1933s.
8. Sithithaworn, P., Haswell-Elkins, M. R., Mairiang, P., Satarug, S., Mairiang, E., Vatanasapt, V., and Elkins, D. B. (1994) *Int. J. Parasitol. 24*, 833−843.
9. Haswell-Elkins, M. R., Satarug, S., Tsuda, M., Mairiang, E., Esumi, H., Sithithaworn, P., Mairiang, P., Saitoh, M., Yongvanit, P., and Elkins, D. B. (1994) *Mutat. Res. 305*, 241−252.
10. Ishii, A., Matsuoka, H., Aji, T., Ohta, N., Arimoto, S., Wataya, Y., and Hayatsu, H. (1994) *Mutat. Res. 305*, 273−281.
11. Harrison, R. J. (1977) in *Textbook of Medicine with Relevant Physiology and Anatomy*, Vol. XXII, Hoddur & Stroughton, London.
12. Klebanoff, S. J. (1980) *Ann. Intern. Med. 93*, 480−489.
13. Harrison, J. E., and Schultz, J. (1976) *J. Biol. Chem. 251*, 1371−1374.
14. Babior, B. M. (1978) *N. Engl. J. Med. 298*, 659−663.
15. Podrez, E. A., Abu-Soud, H. M., and Hazen, S. L. (2000) *Free Radical Biol. Med. 28*, 1717−1725.
16. Mason, R. (1982) in *Free Radicals in Biology* (Pryor, W. A., Ed.) pp 161−222, Academic Press, New York.
17. Cerutti, P. A. (1985) *Science 227*, 375−381.
18. Feig, D. I., Reid, T. M., and Loeb, L. A. (1994) *Cancer Res. 54*, 1890s−1894s.
19. Loft, S., and Poulsen, H. E. (1996) *J. Mol. Med. 74*, 297−312.
20. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915−7922.
21. Babior, B. M., Kipnes, R. S., and Curnutte, J. T. (1973) *J. Clin. Invest. 52*, 741−744.
22. Birnboim, H. C. (1982) *Science 215*, 1247−1249.
23. Shacter, E., Beecham, E. J., Covey, J. M., Kohn, K. W., and Potter, M. (1988) *Carcinogenesis 9*, 2297−2304.
24. Weitberg, A. B. (1989) *Mutat. Res. 224*, 1−4.
25. Weitzman, S. A., Weitberg, A. B., Clark, E. P., and Stossel, T. P. (1985) *Science 227*, 1231−1233.
26. Yamashina, K., Miller, B. E., and Heppner, G. H. (1986) *Cancer Res. 46*, 2396−2401.
27. Barak, M., Ulitzur, S., and Merzbach, D. (1983) *Mutat. Res. 121*, 7−16.
28. Zimmerman, R., and Cerutti, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2085−2087.
29. Shih, K. L., and Lederberg, J. (1976) *Science 192*, 6299−6308.
30. Cozzie, D. A., Kanniganti, R., Charles, M. J., Johnson, J. D., and Ball, L. M. (1993) *Environ. Mol. Mutagen. 21*, 237−246.
31. Trush, M. A., and Kensleer, T. W. (1991) *Free Radical Biol. Med. 10*, 201−209.
32. Tsarta, Y., Subrhmanyam, V. V., Marchall, W., and O'Brien, P. J. (1985) *Chem.-Biol. Interact. 53*, 25−35.
33. Eastmond, D. A., French, R. C., Ross, D., and Smith, M. T. (1987) *Cancer Lett. 35*, 79−86.
34. Eastmond, D. A., Smith, M. T., and Irons, R. D. (1987) *Toxicol. Appl. Pharmacol. 91*, 85−95.
35. London, S. J., Lehman, T. A., and Taylor, J. A. (1997) *Cancer Res. 57*, 5001−5003.
36. Cascorbi, I., Hennings, S., Brockmoller, J., Gephart, J., Meisel, C., Muller, J. M., Loddenkemper, R., and Roots, I. (2000) *Cancer Res. 60*, 644−649.

37. Imlay, J. A., Chin, S. M., and Linn, S. (1988) *Science 240*, 640−642.
38. Fenton, H. J. H. (1894) *J. Chem. Soc. 65*, 899−910.
39. Haber, F., and Weiss, J. (1932) *Naturwissenschaften 20*, 948−950.
40. Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) *J. Clin. Invest. 70*, 598−607.
41. Klebanoff, S. J., Waltersdorph, A. M., and Rosen, H. (1984) *Methods Enzymol. 105*, 399−403.
42. Albrich, J. M., McCarthy, C. A., and Hurst, J. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 210−214.
43. Nauseef, W. M. (1988) *Hematol. Oncol. Clin. North Am. 2*, 135−158.
44. Shen, Z., Wu, W., and Hazen, S. L. (2000) *Biochemistry 39*, 5474−5482.
45. Candeias, L. P., Patel, K. B., Stratford, M. R., and Wardman, P. (1993) *FEBS Lett. 333*, 151−153.
46. Long, C. A., and Bielski, B. H. T. (1980) *J. Phys. Chem. 84*, 555−557.
47. Ross, A. B., Mallard, N. G., Helman, W. P., Bielski, B. H. T., and Buxton, G. V. (1999) *NDRL-NIST Solution Kinetics Database, version 1*.
48. Henderson, J. P., Byun, J., and Heinecke, J. W. (1999) *J. Biol. Chem. 274*, 33440−33448.
49. Shigenaga, M. K., Park, J. W., Cundy, K. C., Gimeno, C. J., and Ames, B. N. (1990) *Methods Enzymol. 186*, 521−530.
50. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. (1992) *J. Biol. Chem. 267*, 166−172.
51. Kamiya, H., Miura, H., Murata-Kamiya, N., Ishikawa, H., Sakaguchi, T., Inoue, H., Sasaki, T., Masutani, C., Hanaoka, F., Nishimura, S., et al. (1995) *Nucleic Acids Res. 23*, 2893−2899.
52. Byun, J., Henderson, J. P., Mueller, D. M., and Heinecke, J. W. (1999) *Biochemistry 38*, 2590−2600.
53. Dizdaroglu, M. (1991) *Free Radical Biol. Med. 10*, 225−242.
54. Chung, F. L., Young, R., and Hecht, S. S. (1984) *Cancer Res. 44*, 990−995.
55. Simic, M. G. (1994) *Cancer Res. 54*, 1918s−1923s.
56. Spry, C. J. F. (1988) *Eosinophils. A Comprehensive Review and Guide to the Medical Literature*, Oxford University Press, Oxford, U.K.
57. Rothenberg, M. E. (1998) *N. Engl. J. Med. 338*, 1592−1600.
58. Wardlaw, A. J. (1994) *Postgrad. Med. J. 70*, 536−552.
59. Oakley, G. G., Devanaboyina, U., Robertson, L. W., and Gupta, R. C. (1996) *Chem. Res. Toxicol. 9*, 1285−1292.
60. Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D., and Regiania, S. (1986) *Science 234*, 200−203.
61. Wu, W., Samoszuk, M. K., Comhair, S. A. A., Thomassen, M. J., Farver, C. F., Dweik, R. A., Kavuru, M. S., Erzurum, S. C., and Hazen, S. L. (2000) *J. Clin. Invest. 105*, 1455−1463.
62. Jorg, A., Pasquier, J. M., and Klebanoff, S. J. (1982) *Biochim. Biophys. Acta 701*, 185.
63. van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J. (1997) *Biochem. J. 327*, 487−492.
64. Rakita, R. M., Michel, B. R., and Rosen, H. (1990) *Biochemistry 29*, 1075−1080.
65. Thomas, E. L., Bates, K. P., and Jefferson, M. M. (1981) *J. Dent. Res. 60*, 785−796.
66. Agner, K. (1963) *Acta Chem. Scand. 17*, S332−S338.
67. Bolscher, B. G., Plat, H., and Wever, R. (1984) *Biochim. Biophys. Acta 784*, 177−186.
68. Carlson, M. G., Peterson, C. G., and Venge, P. (1985) *J. Immunol. 134*, 1875−1879.
69. Wajon, J. E., and Morris, J. C. (1980) in *Water chlorination: Environmental impact and health effects* (Jolley, R. L., Brungs, W. A., Cumming, R. B., and Jacobs, V. A., Eds.) pp 171−181, Ann Arbor Science Publishers Inc., Ann Arbor, MI.
70. Wajon, J. E., and Morris, J. C. (1982) *Inorg. Chem. 21*, 4258−4263.
71. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
72. Thomas, E. L., Bozeman, P. M., Jefferson, M. M., and King, C. C. (1995) *J. Biol. Chem. 270*, 2906−2913.
73. van der Vleit, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) *J. Biol. Chem. 272*, 7617−7625.
74. Rice, J. M., and Dudek, G. O. (1967) *J. Am. Chem. Soc. 89*, 2719−2725.
75. Wu, W., Chen, Y., d'Avignon, A., and Hazen, S. L. (1999) *Biochemistry 38*, 3538−3548.
76. Hazen, S. L., Hsu, F. F., Duffin, K., and Heinecke, J. W. (1996) *J. Biol. Chem. 271*, 23080−23088.
77. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) *J. Clin. Invest. 98*, 1283−1289.
78. O'Neill, J. P., Heartlein, M. W., and Preston, R. J. (1983) *Mutat. Res. 109*, 259−270.
79. Tavale, S. S., and Sobell, H. M. (1970) *J. Mol. Biol. 48*, 109−123.
80. Quigley, G. J., van der Marel, G. A., van Boom, J. H., Wang, A. H. J., and Rich, A. (1986) *Science 232*, 1255−1258.

BI001961T