Activated Leukocytes Oxidatively Damage DNA, RNA, and the Nucleotide Pool through Halide-Dependent Formation of Hydroxyl Radical†

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Received December 7, 1999

ABSTRACT: A variety of chronic inflammatory conditions are associated with an increased risk for the development of cancer. Because of the numerous links between DNA oxidative damage and carcinogenesis, a potential role for leukocyte-generated oxidants in these processes has been suggested. In the present study, we demonstrate a novel free transition metal ion-independent mechanism for hydroxyl radical (•OH)-mediated damage of cellular DNA, RNA, and cytosolic nucleotides by activated neutrophils and eosinophils. The mechanism involves reaction of peroxidase-generated hypohalous acid (HOCl or HOBr) with intracellular superoxide ($O_2^{\bullet-}$) forming •OH, a reactive oxidant species implicated in carcinogenesis. Incubation of DNA with either isolated myeloperoxidase (MPO) or eosinophil peroxidase (EPO), plasma levels of halides (Cl^- and Br^-), and a cell-free $O_2^{\bullet-}$ -generating system resulted in DNA oxidative damage. Formation of 8-hydroxyguanine (8-OHG), a mutagenic base which is a marker for •OH-mediated DNA damage, required peroxidase and halides and occurred in the presence of transition metal chelators (DTPA ± desferrioxamine), and was inhibited by catalase, superoxide dismutase (SOD), and scavengers of hypohalous acids. Similarly, exposure of DNA to either neutrophils or eosinophils activated in media containing metal ion chelators resulted in 8-OHG formation through a pathway that was blocked by peroxidase inhibitors, hypohalous acid scavengers, and catalytically active (but not heat-inactivated) catalase and SOD. Formation of 8-OHG in target cells (HA1 fibroblasts) occurred in all guanyl nucleotide-containing pools examined following exposure to both a low continuous flux of HOCl (at sublethal doses, as assessed by [^{14}C]adenine release and clonogenic survival), and hyperoxia (to enhance intracellular $O_2^{\bullet-}$ levels). Mitochondrial DNA, poly A RNA, and the cytosolic nucleotide pool were the primary targets for oxidation. Moreover, modest but statistically significant increases in the 8-OHG content of nuclear DNA were also noted. These results suggest that the peroxidase−H_2O_2−halide system of leukocytes is a potential mechanism contributing to the well-established link between chronic inflammation, DNA damage, and cancer development.

The contribution of oxidative processes to carcinogenesis is now widely accepted (*1*−*7*). Much progress in this area involves use of stable markers of free-radical reactions to identify specific chemical mechanisms of DNA damage in vivo (*8*−*11*). DNA damage caused by the highly reactive hydroxyl radical (•OH)[1] has been linked to neoplasia in a number of biological systems (*1*−*12*).

DNA damage by •OH generates characteristic mutagenic base lesions, such as 8-hydroxyguanine (8-OHG) (*8*−*12*). Significant progressive increases in the content of •OH-generated lesions in the DNA from normal, premalignant, cancerous, and metastatic tissues have been demonstrated utilizing a variety of independent analytical methods (*5*−*7*, *12*, *13*). The direct correlation observed between free radical-mediated 8-OHG formation and carcinogenesis has been attributed to the ability of this modified base to induce mutagenic events during DNA synthesis (*14*, *15*). The pathways responsible for generating •OH in vivo are unclear. Free metal ions, like Fe^{2+} or Cu^{2+}, are thought to play a role because of their well-known ability to catalyze conversion of reduced oxygen species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) into the more reactive •OH (eq 1) (*16*):

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

Numerous sources of $O_2^{\bullet-}$ and H_2O_2 during carcinogenesis have been suggested, including redox cycling of estrogens (*17*, *18*), xenoestrogens (*19*, *20*), and xenobiotics such as polychlorinated biphenyls (*21*), peroxidase-oxidized products (*22*, *23*), and a variety of intracellular sources (*24*, *25*). The

† This work was supported in part by a grant from the Department of Defense (BC980289) and by grants from the National Institutes of Health (HL62526 and HL29582). Z.S. is a recipient of a Jane Coffin Childs Memorial Fund for Medical Research Fellowship.

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[1] Abbreviations: 8-OHG, 8-hydroxyguanine; Atz, aminotriazole; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; EPO, eosinophil peroxidase; G, guanine; HBSS, Hanks balanced salt solution; HOCl, hypochlorous acid; HOBr, hypobromous acid; H_2O_2, hydrogen peroxide; HPLC-EC, high-performance liquid chromatography with electrochemical detection; MPO, myeloperoxidase; $O_2^{\bullet-}$, superoxide anion; •OH, hydroxyl radical; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

10.1021/bi992809y CCC: $19.00
Published on Web 04/14/2000

mechanism(s) responsible for generating $^{\bullet}OH$ during the development of cancer and metastases formation in vivo is (are) still unclear.

Phagocytic cells such as neutrophils and eosinophils are a particularly attractive source of reactive intermediates since they have evolved enzymatic mechanisms to generate an arsenal of reactive species as part of their normal function. Neutrophils and eosinophils play an essential role in tissue surveillance in host defense mechanisms. These cells inflict oxidative damage upon invading parasites and pathogens; however, the reactive species they form can also damage normal tissues (*26*−*30*). Activated neutrophils and eosinophils employ NADPH oxidase to catalyze conversion of molecular oxygen to $O_2^{\bullet-}$, which dismutates to form H_2O_2 (*31*). Research thus far into the mechanisms of phagocyte-dependent DNA damage has primarily focused on the potential role(s) of $O_2^{\bullet-}$ and H_2O_2 in DNA damage through metal ion-dependent generation of $^{\bullet}OH$, or in the oxidative conversion of precarcinogens into carcinogens (*4*, *32*−*37*). In addition to its ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) (the presumed catalyst involved in formation of $^{\bullet}OH$), a role for $O_2^{\bullet-}$ in releasing iron from protein-bound iron−sulfur clusters has also been suggested (*38*).

An alternative pathway for $^{\bullet}OH$ formation in vivo may involve myeloperoxidase (MPO) and eosinophil peroxidase (EPO), abundant heme proteins secreted by neutrophils and eosinophils, respectively. MPO and EPO amplify the oxidative potential of H_2O_2 by generating reactive halogen (*39*−*43*), reactive nitrogen (*44*−*49*), reactive aldehyde (*28*, *50*, *51*), and diffusible radical species (*45*, *52*, *53*). Under physiological concentrations of halides, major initial oxidants formed by MPO and EPO are hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively. Studies with spin trapping agents (*54*, *55*) and chemical traps of $^{\bullet}OH$ (*56*, *57*) have demonstrated that hypohalous acids can generate $^{\bullet}OH$ following reaction with $O_2^{\bullet-}$ (eq 2):

$$O_2^{\bullet-} + HOX \rightarrow {}^{\bullet}OH + X^- + O_2 \ (X = Cl \text{ or } Br) \quad (2)$$

This reaction is analogous to the Haber−Weiss reaction (eq 1) where H_2O_2 is replaced by HOCl or HOBr. In the absence of metal ions, eq 2 is at least 6 orders of magnitude faster than the Haber−Weiss reaction (*57*−*59*).

In the present study, we examine the potential role of leukocyte-generated hypohalous acids and intracellular $O_2^{\bullet-}$ as mediators of $^{\bullet}OH$-dependent damage of cellular DNA, RNA, and free nucleotides. We now demonstrate that exposure of DNA to either isolated MPO or EPO and a $O_2^{\bullet-}$-generating system results in $^{\bullet}OH$-mediated DNA damage through a free metal ion-independent, halide-dependent pathway. We also demonstrate that activated neutrophils and eosinophils oxidatively damage DNA through this pathway. Finally, we demonstrate that exposure of target cells to both an exogenous source of hypohalous acids and enhanced intracellular $O_2^{\bullet-}$ production results in significant $^{\bullet}OH$-mediated damage of nuclear DNA, mitochondrial DNA, RNA, and cytosolic nucleotides.

EXPERIMENTAL PROCEDURES

Materials

Organic solvents (HPLC-grade) and H_2O_2 (30%; ACS grade) were obtained from Fisher Chemical Co. (Pittsburgh, PA). 2-Deoxy-8-hydroxyguanosine (8-OHdG) was obtained from Sigma Chemical Co. (St. Louis, MO). Chelex-100 resin (200−400 mesh, sodium form) was obtained from BioRad (Hercules, CA). 8-Hydroxyguanine (8-OHG) and 8-[1,2-^{3}H]-OHG were prepared as described (*9*). DNA Extractor WB kits were obtained from Wako Chemical (Richmond, VA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Methods

General Procedures. Homogeneous human MPO and porcine EPO were isolated and quantified as described (*43*). Rat liver DNA was extracted and isolated as described (*60*). Neutrophils and eosinophils were isolated by buoyant density centrifugation (*61*). Low levels of contaminating eosinophils or neutrophils in neutrophil and eosinophil preparations, respectively, were then removed by cell sorting (*62*). Buffers were prepared with distilled deionized water. To remove any remaining potential adventitious metals that could participate in the generation of $^{\bullet}OH$, all reagents, enzymes, and buffers were treated with chelex-100 resin, and reactions were performed in the presence of the metal chelator diethylenetriaminepentaacetic acid (DTPA). All reaction vials, pipet tips, and pH electrodes were rinsed with 100 μM DTPA (pH 7.0) prior to use.

DNA Modification. A stock solution of calf thymus DNA (1 mg/mL) in 20 mM sodium phosphate buffer (pH 7.0) was treated with chelex-100 resin and centrifuged to remove resin, and the pH was adjusted to 7.0 using chelex-treated monobasic sodium phosphate buffer (20 mM) prior to use. If not used immediately, stock solutions were sparged and stored under argon in gastight brown glass vials. Typically, calf thymus DNA (0.8 mg/mL final) was incubated with chelex-treated MPO or EPO (100 nM), a cell-free superoxide generating system comprised of lumazine (0.4 mM), and chelex-treated bovine milk xanthine oxidase (XO, 80 milliunits/mL; Boehringer Mannheim) in the presence or absence of halides. Mixtures were incubated at 37 °C for 1 h, and the reaction was stopped by extraction of DNA using the NaI chaotropic method (*60*).

DNA modification by isolated human neutrophils or human eosinophils was performed by incubating freshly isolated chelex-treated rat liver DNA (0.8 mg/mL final) with cells (5×10^5 cells/mL) in chelex-treated Hank's balanced salt solution (HBSS) supplemented with DTPA (pH 7.4) and plasma levels of Br^- (i.e., 100 μM NaBr). Leukocytes were stimulated with 200 nM phorbol myristate acetate (PMA). In some cases, the suspensions also contained one of the following: 500 units/mL chelex-treated bovine liver catalase, 30 units/mL chelex-treated superoxide dismutase (SOD), 1 mM azide, 10 mM aminotriazole (ATZ), or 100 μM desferrioxamine. Following incubation, cells were pelleted, and the DNA in supernatants was extracted and analyzed for 8-OHG content.

DNA modification of target cells was done using Chinese hamster fibroblasts (HA1 cell line). HA1 cells were maintained in Eagle's minimum essential medium supplemented with fetal calf serum and penicillin−streptomycin (100 units/mL−0.1 mg/mL) in a humidified 5% CO_2 and air environment. Cells were placed in HBSS supplemented with DTPA (100 μM) during the 4 h treatments. The exogenous HOCl-

generating system used was comprised of MPO (100 nM), glucose (in media)/glucose oxidase (10 ng/mL), and Cl^- (in media). Under these conditions, a continuous flux of HOCl of 0.08 μM/min is produced, as assessed by the taurine monochloramine method (*63*), in HBSS supplemented with 10 mM taurine. In some experiments, HA1 cells were maintained in an incubator at an oxygen concentration to 95% as the 4 h hyperoxia condition as described (*64*). Following the 4 h reaction period at 37 °C, cells from multiple dishes were recovered, pooled and pelleted, and washed with ice-cold HBSS supplemented with 100 μM DTPA and butylated hydroxytoluene (BHT, 50 μM), and the content of 8-OHG in at least 20 μg of nuclear DNA, mitochondrial DNA, poly A RNA, and cytosolic nucleotides was then determined by reverse-phase HPLC-EC.

HA1 cellular homogenate was generated in chelex-treated sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) and BHT (50 μM). Nuclei and mitochondria were isolated by conventional subcellular fractionation methods prior to isolation of DNA by the NaI chaotropic method (*60*). RNA was isolated as described by Fiala (*65*) with the addition of BHT (100 μM) and desferol (100 μM) to all solutions. Protein in cytosol (100,000*g* supernatant of homogenate) was precipitated by trichloroacetic acid (30% v/v, 0 °C). The samples were spun at 2000*g*, and the resultant supernatant was passed through a DTPA (100 μM)-rinsed 3000 kDa molecular mass cutoff filter and used for analysis of 8-OHG content in the cytosolic free pool. DNA, RNA, and the low molecular weight cytosolic fraction were alternately vacuum-degassed and purged with argon 5 times. Samples were then incubated with formic acid (60% v/v) at 130 °C for 45 min under a blanket of argon. Hydrolysates were resuspended in 200 μL of chelex-treated H_2O and subjected to HPLC-EC analysis.

Reverse Phase HPLC-EC Quantification of 8-OHG. The contents of 8-OHG and guanine (G) in hydrolysates were determined by reverse phase HPLC-EC using an ESA (Cambridge, MA) CoulArray HPLC instrument equipped with 8 electrochemical cells (channels) arranged in series and set to increasing specified potentials as follows: channel 1 (100 mV); channel 2 (250 mV); channel 3 (290 mV); channel 4 (350 mV); channel 5 (480 mV); channel 6 (580 mV); channel 7 (640 mV); and channel 8 connected to a variable UV−Vis detector. Samples were injected onto a Progel TSK ODS-AD TM column (5 μm, 4.6 × 250 mm) equilibrated with mobile phase A (15 mM lithium phosphate, 3 mg/L lithium dodecyl sulfate, pH 3.2). Products were eluted at a flow rate of 1 mL/min with a nonlinear gradient generated with mobile phase B (50% methanol, 15 mM lithium phosphate 3 mg/L lithium dodecyl sulfate, pH 3.2) as follows: isocratic solution at 0% mobile phase B for 30 min, 0−25% mobile phase B over 60 min, 25−100% mobile phase B for 2 min, and isocratic elution at 100% mobile phase B for 20 min. In preliminary studies, peak identity as 8-OHG was established by reverse phase HPLC with on-line electrospray mass spectrometry. Recoveries were also established by spiking samples with high specific activity ^{3}H-labeled 8-OHG standard and then collecting the final peak of interest and counting recovered mass with a scintillation counter. Peak identity was subsequently routinely established by demonstrating the appropriate retention time, redox potential, and ratio of integrated currents in adjacent channels. The content of guanine (G) was determined by UV monitoring at 256 nm. Authentic 8-OHG and G standards were also used to generate external calibration curves.

Measurement of Cellular Injury and Clonogenic Survival Experiments. The degree of cellular injury was measured by the specific release of ^{14}C from proliferating cells previously loaded with ^{14}C-labeled adenine as described (*66*). Clonogenic survival experiments were performed in 60 mm dishes and grown exponentially for 2 days, at which time the cell density was approximately $5 \times 10^4/cm^2$ as described (*64*). During survival experiments, cultures were treated for 4 h with 4 mL of the exposure indicated in Table 1. Cultures were then washed 3 times with sterile Puck's saline and trypsin-treated, and the resulting single-cell suspension was counted (by Coulter counter). Suspensions were serially diluted and replicates plated for colony survival. After 8−10 days incubation at 37 °C, colonies were fixed in 70% ethanol, stained with Crystal Violet, and counted under a dissecting microscope. Plating efficiencies were approximately 70−85% for all untreated cells.

Statistical Analysis. Differences between treated groups were determined using the paired Student's *t* test assuming a normal distribution. Significance levels were set at $\alpha = 0.05$ for two-tailed tests. When multiple comparisons were made, a Bonferoni correction to the significance criterion for each test was made.

RESULTS

MPO and EPO Promote $^{\bullet}$OH-Mediated Damage of DNA. Calf thymus DNA was incubated in the presence of a cell-free $O_2^{\bullet-}$ -generating system (lumazine/xanthine oxidase) in chelex-100-treated sodium phosphate buffer (pH 7.0) supplemented with the metal chelator DTPA and plasma levels of halides (100 mM Cl^- and 100 μM Br^-). DNA in the reaction mixture was then precipitated and hydrolyzed, and the content of 8-OHG was determined by HPLC-EC as described under Experimental Procedures. In the absence of the leukocyte peroxidases, small but detectable peaks with identical retention time and electrochemical potential to those of authentic 8-OHG were observed (Figure 1, "− MPO" and "− EPO" chromatograms). The modest levels of 8-OHG present under these conditions were similar to those observed in untreated DNA (Figure 2) and were consistent with background levels of the adduct in commercial preparations of calf thymus DNA, as previously reported (*60, 67*). Upon addition of either purified chelex-treated MPO or EPO to the reaction mixtures, significant levels of the $^{\bullet}$OH-dependent product 8-OHG were formed (Figure 1, "+ MPO" and "+ EPO" chromatograms, and Figure 2). Importantly, formation of the mutagenic base upon addition of either MPO or EPO was halide-dependent (Figure 2), consistent with $^{\bullet}$OH formation by interaction of $O_2^{\bullet-}$ and a peroxidase-generated hypohalous acid (eq 2). For example, in mixtures containing isolated MPO, the content of 8-OHG formed was dramatically attenuated by omission of Cl^-, a major substrate of MPO (*39*), despite the presence of physiological levels of Br^-. Likewise, omission of Br^-, a preferred substrate of EPO (*42*), from DNA mixtures containing EPO, a $O_2^{\bullet-}$ -generating system, and plasma levels of Cl^-, resulted in a significant reduction in the extent of 8-OHG formed (Figure 2). Finally, omission of xanthine oxidase from reaction mixtures ablated 8-OHG formation (Figure 2).

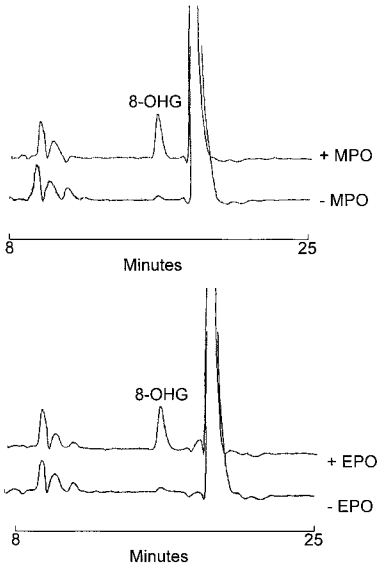

FIGURE 1: Reverse phase HPLC-EC detection of 8-OHG in DNA exposed to the MPO (top panel) and EPO (bottom panel) systems. Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 3 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), NaCl (100 mM), NaBr (100 μM), and a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase) in the absence ("− MPO" and "− EPO") or presence ("+ MPO" and "+ EPO") of either MPO or EPO (105 nM each). DNA was then precipitated, washed, hydrolyzed, and then analyzed by reverse phase HPLC-EC as described under Experimental Procedures. Full scale = 50 nA. EPO, eosinophil peroxidase; MPO, myeloperoxidase; 8-OHG, 8-hydroxyguanine.

Mechanism of 8-OHG Formation during Exposure of DNA to MPO and EPO. To further explore the mechanism of DNA damage by the $^{\bullet}$OH-like oxidant formed by MPO and EPO, we first evaluated the halide concentration-dependence of 8-OHG formation. Each isolated peroxidase was individually incubated with DNA, a cell-free $O_2^{\bullet-}$ -generating system, and differing concentrations of either Cl^- or Br^- in chelex-100-treated buffer supplemented with DTPA, and the 8-OHG content of DNA was then determined by HPLC-EC analysis. Dose-dependent formation of 8-OHG by physiologically relevant levels of Cl^- and Br^- in reaction mixtures containing MPO and EPO, respectively, was observed (Figure 3). Moreover, addition of desferrioxamine, a chelator which potently blocks iron ion-dependent formation of $^{\bullet}$OH, did not completely block 8-OHG formation. In contrast, 8-OHG production was inhibited by addition of methionine, a scavenger of hypohalous acids (*63*), as well as either catalase or SOD, scavengers of H_2O_2 and $O_2^{\bullet-}$, respectively (Figure 3). The present results thus distinguish $^{\bullet}$OH-dependent DNA damage mediated by MPO and EPO from classic free transition metal ion-catalyzed Fenton and Haber Weiss reactions; rather than being inhibited by metal chelators, peroxidase-dependent formation of $^{\bullet}$OH likely occurs through secondary oxidation reactions between a hypohalous acid and $O_2^{\bullet-}$.

Activated Neutrophils and Eosinophils Promote $^{\bullet}$OH-Mediated DNA Damage through Interaction of a Peroxidase-Generated Hypohalous Acid and $O_2^{\bullet-}$. Exposure of calf thymus DNA to either activated neutrophils or eosinophils in chelex-100-treated media supplemented with DTPA resulted in modest increases in 8-OHG (data not shown).

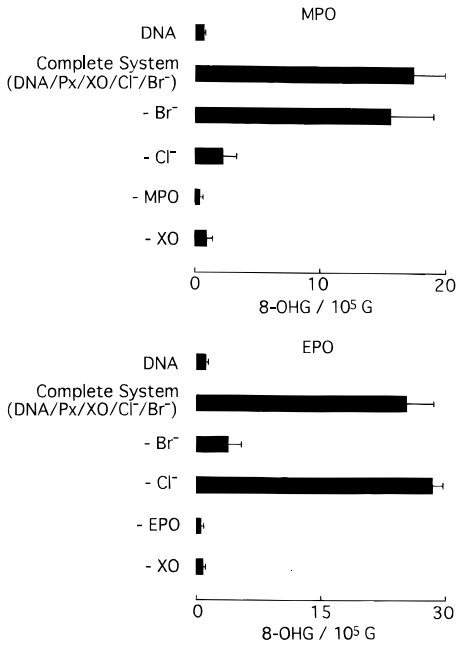

FIGURE 2: Reaction requirements for 8-OHG formation in DNA exposed to MPO (top panel) and EPO (bottom panel). Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), NaCl (100 mM), NaBr (100 μM), and a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase) in the presence of 100 nM MPO (top panel, Complete System) or EPO (bottom panel, Complete System). Additions or deletions to the Complete Systems were as indicated. Following reaction, DNA was precipitated, washed, and hydrolyzed, and the content of 8-OHG present was determined by HPLC-EC as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. EPO, eosinophil peroxidase; G, guanine; MPO, myeloperoxidase; 8-OHG, 8-hydroxyguanine; Px, peroxidase; XO, xanthine oxidase.

However, these increases were difficult to detect reproducibly because of the relatively high background level of 8-OHG in commercial preparations of calf thymus DNA. We therefore subsequently used freshly isolated rat liver DNA as a target for cell-dependent oxidation reactions, since it possessed at least 10-fold lower background levels of 8-OHG. Incubation of rat liver DNA with either PMA-stimulated neutrophils or eosinophils generated significant levels of 8-OHG (Figure 4). 8-OHG formation required cell activation, was only modestly attenuated by addition of desferrioxamine, and was blocked by addition of peroxidase inhibitors (aminotriazole or NaCN) and hypohalous acid scavengers (methionine) (Figure 4). Both SOD and catalase, but neither heat-inactivated SOD nor heat-inactivated catalase, dramatically inhibited 8-OHG production, indicating that both $O_2^{\bullet-}$ and H_2O_2 were required for leukocyte-dependent 8-OHG formation under these conditions. Collectively, these results are consistent with a role for $O_2^{\bullet-}$ and a peroxidase−H_2O_2−halide system—not a free transition metal ion-dependent pathway—in promoting 8-OHG generation by the leukocytes.

Formation of Intracellular 8-OHG following Exposure of Cells to a Nonlethal Flux of HOCl. Experiments thus far described were designed to identify a free metal ion-independent mechanism of 8-OHG formation in isolated DNA exposed to either purified peroxidase−H_2O_2−halide systems or isolated activated human leukocytes. To further assess the potential pathophysiological significance of this

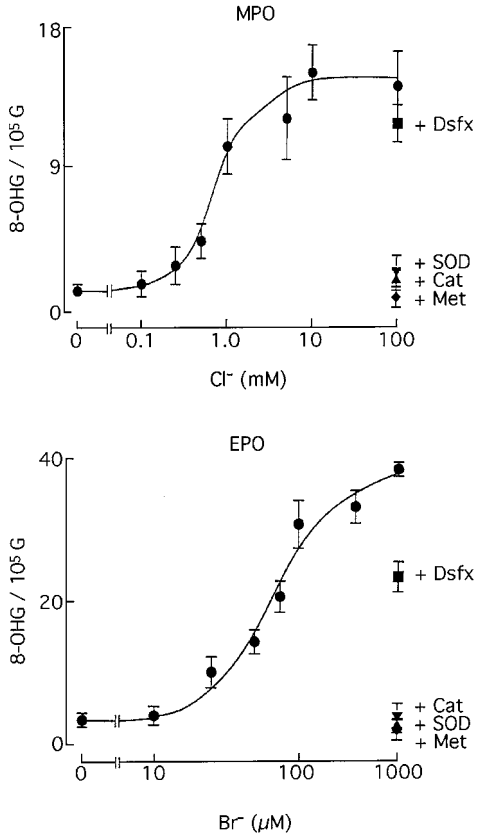

FIGURE 3: Halide concentration dependence of 8-OHG formation by MPO (top panel) and EPO (bottom panel). (Top panel) Calf thymus DNA (400 μg, 0.8 mg/mL final) was incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), 100 nM MPO, a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase), and the indicated concentration of NaCl. In parallel (bottom panel), calf thymus DNA (400 μg, 0.8 mg/mL final) was also incubated for 4 h at 37 °C in sodium phosphate buffer (20 mM, pH 7.0) supplemented with DTPA (100 μM), 100 mM NaCl, 100 nM EPO, a cell-free $O_2^{\bullet-}$ generating system (0.4 mM lumazine + 80 milliunits/mL xanthine oxidase), and the indicated concentration of NaBr. The content of 8-OHG in DNA was then determined by reverse phase HPLC-EC as described under Experimental Procedures. Where indicated, desferrioxamine (100 μM), SOD (30 units/mL), bovine liver catalase (500 units/mL), or methionine (100 μM) were included during reactions. Data represent the mean ± SD of triplicate determinations. Cat, catalase; Dsfx, desferrioxamine; SOD, superoxide dismutase; Met, methionine.

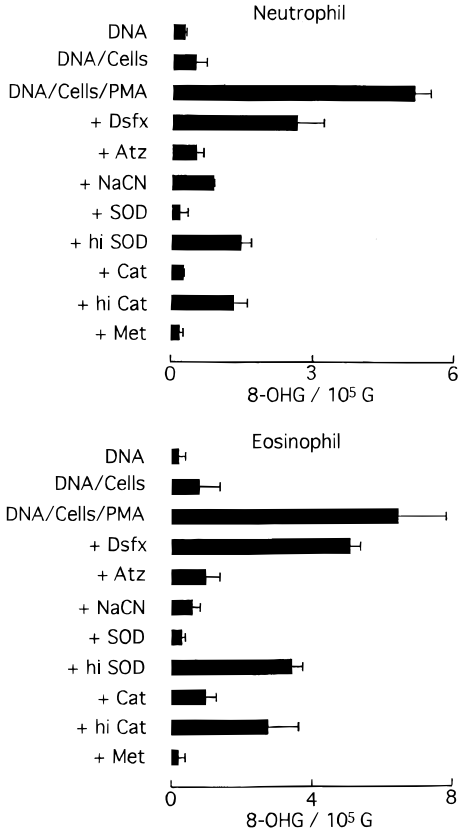

FIGURE 4: 8-OHG formation in rat liver DNA by stimulated neutrophils (top panel) and eosinophils (bottom panel). Rat liver DNA (400 μg, 0.8 mg/mL final) was incubated with the indicated additions in HBSS (pH 7.4) supplemented with DTPA (100 μM) and NaBr (100 μM) for 90 min at 37 °C. Where indicated, (top panel) neutrophils or (bottom panel) eosinophils (5×10^5 cells/mL) were included. In some cases, the suspensions also contained phorbol myristate acetate (200 nM), bovine liver catalase (500 units/mL), superoxide dismutase (30 units/mL), NaCN (1 mM), aminotriazole (10 mM), or desferrioxamine (100 μM). Following incubation, cells were pelleted, and DNA in supernatants was recovered and analyzed for 8-OHG content by HPLC-EC as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. Atz, 3-aminotriazole; Cat, catalase; hi Cat, heat-inactivated catalase; hi SOD, heat-inactivated superoxide dismutase; Met, methionine; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

pathway for DNA damage, we examined whether intracellular 8-OHG could be formed following exposure of target cells to a sublethal flux of hypohalous acids. Such conditions are likely to mimic those present in vivo at sites of chronic inflammation. HA1 fibroblasts incubated under control conditions (4 h in chelex-100-treated HBSS supplemented with 100 μM DTPA under 5% CO_2, 95% air) contained low but detectable levels of 8-OHG in nuclear DNA (Table 1) at levels comparable to those reported in other cells (*60*). Increased levels of 8-OHG (relative to nuclear DNA) were observed in mitochondrial DNA, and to a lesser degree poly A RNA and the cytosolic free nucleotide pool, of control cells (Table 1). These results are consistent with studies identifying a high steady-state oxidation level in mitochondrial DNA relative to DNA from the protected environment of the nucleus (*68*). Exposure of HA1 cells to a low continuous flux of HOCl for 4 h resulted in a significant increase in the 8-OHG content of mitochondrial DNA (Table 1). These results suggest that exogenous HOCl may interact with $O_2^{\bullet-}$ generated by mitochondria to promote $^{\bullet}OH$-dependent damage of mitochondrial DNA.

To further explore the potential role of DNA damage by hypohalous acid-generated $^{\bullet}OH$, we examined conditions where intracellular production of $O_2^{\bullet-}$ might be increased. Hyperoxia was chosen because it is a nonpharmacological method of enhancing intracellular production of $O_2^{\bullet-}$ (*69*). Brief exposure (4 h) of HA1 cells to hyperoxic conditions resulted in no significant increase in 8-OHG content. However, cells exposed to a brief period of both hyperoxia and the HOCl-generating system resulted in marked increases in the 8-OHG content of mitochondrial DNA, poly A RNA, and cytosolic nucleotides (Table 1). Small but statistically significant increases in the level of 8-OHG in nuclear DNA were noted as well (Table 1). The mechanism of intracellular 8-OHG formation in HA1 cells was consistent with the requirement for HOCl generation by the MPO−H_2O_2−Cl^- system since omission of either MPO or glucose oxidase from the culture medium resulted in a marked decrease in

Table 1: Intracellular 8-OHG Formation after Brief Exposure to HOCl and Hyperoxia[a]

exposure (4 h)	poly A RNA (8-OHG/10^5 G)	mitochondrial DNA (8-OHG/10^5 G)	cytosolic pool (8-OHG/10^5 G)	nuclear DNA (8-OHG/10^5 G)	specific [^{14}C]adenine release (%)	clonogenic survival[d] (%)
control[b]	1.3 ± 0.9	5.2 ± 1.1	1.0 ± 0.9	0.33 ± 0.07	0	100
(A) MPO/GOx	1.4 ± 0.2	7.2 ± 1.4**	1.4 ± 0.2	0.40 ± 0.14	26	85 ± 3
(B) hyperoxia[c]	2.0 ± 0.4	6.9 ± 2.5	1.6 ± 0.4	0.31 ± 0.04	<5	98 ± 5
(A + B) complete	7.2 ± 3.0***	75.3 ± 6.2***	8.1 ± 1.3***	0.57 ± 0.22*	38	82 ± 4
−MPO	2.7 ± 1.8	7.6 ± 3.7	1.7 ± 0.8	0.41 ± 0.14	7	96 ± 5
−GOx	0.8 ± 0.3	5.9 ± 0.9	0.8 ± 0.3	0.31 ± 0.13	<5	99 ± 4

[a] Chinese hamster fibroblasts (HA1 cell line) were incubated for 4 h at 37 °C in 5% CO_2, 95% air in media alone (control); in the presence of an exogenous HOCl-generating system (condition A); in 95% O_2, 5% CO_2 (condition B); in the presence of both a HOCl-generating system and hyperoxia (complete system, conditions A + B); or under conditions of the complete system in the absence of either MPO or glucose oxidase (GOx) as described under Experimental Procedures. Cells were then recovered, and the 8-OHG contents of nuclear DNA, mitochondrial DNA, poly A RNA, and the cytosolic pool were determined as described under Experimental Procedures. In parallel incubations, the specific release of [^{14}C]adenine and clonogenic cell survival assays were performed as described under Experimental Procedures. Data represent the mean ± SD of triplicate determinations. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$ vs control. [b] 5% CO_2 and 95% air. [c] 5% CO_2 and 95% O_2. [d] Normalized to plating efficiency of 76%.

the 8-OHG content of DNA, RNA, and cytosolic nucleotides (Table 1). A mitochondrial source of $O_2^{\bullet-}$ generation during hyperoxia seems likely given the markedly enhanced levels of 8-OHG in mitochondrial DNA noted.

Hypohalous acids are potent cytotoxins (*26*). Moreover, exposure of HA1 cells to prolonged (>40 h) hyperoxia is known to promote cell death (*64*). It was therefore important to establish that the brief exposure to hyperoxia and low levels of HOCl used did not result in significant toxicity and cell lysis. Initial studies examining lactate dehydrogenase (LDH) release into media were abandoned because control studies demonstrated that HOCl can inhibit LDH activity. We therefore screened for cell toxicity (and membrane leak) by quantifying the specific release of radioactivity from HA1 cells preloaded with ^{14}C-labeled adenine (*66*). In parallel, we examined the mitotic competence of treated cells using clonogenic cell survival assays (*64*). Under the conditions employed, brief exposure of HA1 cells to hyperoxia failed to result in any detectable injury, as assessed by either specific [^{14}C]adenine release or clonogenic cell survival (Table 1). Exposure of cells (4 h) to the low flux of HOCl employed (Table 1, condition "A") resulted in 26% specific [^{14}C]adenine release while 85% of cells remained clonogenically competent. HA1 cells exposed to both hyperoxia and the exogenous HOCl-generating system under conditions identical to those which produced significant levels of intracellular 8-OHG (Table 1, conditions "A + B") resulted in release of 38% of preloaded [^{14}C]adenine while demonstrating an overall clonogenic survival of 82%. These results demonstrate that the brief duration of hyperoxia and modest levels of HOCl employed are not lethal to the majority of cells. They also suggest that a significant portion of HA1 cells initially injured possess adequate repair mechanisms to survive and subsequently undergo mitosis.

DISCUSSION

In this study we investigated the ability of peroxidase−H_2O_2 systems of leukocytes to promote DNA oxidative damage through halide-dependent formation of $^{\bullet}$OH. Multiple lines of evidence support a role for peroxidase-generated hypohalous acids and $O_2^{\bullet-}$ in promoting $^{\bullet}$OH production (eq 2) and subsequent DNA damage. In the absence of free transition metal ions, DNA incubated in the presence of a $O_2^{\bullet-}$ generating system was not significantly modified, as assessed by nominal levels of 8-OHG (Figure 1). However, when isolated MPO or EPO was added to reaction mixtures, 8-OHG was readily detected (Figure 1). Thus, formation of the $^{\bullet}$OH-generated product was dependent on the presence of a peroxidase. A critical role for hypohalous acid generation was confirmed to be a necessary intermediate in 8-OHG formation by demonstrating an absolute halide-dependence for the reaction (Figures 2 and 3). Physiological levels of chloride and bromide were utilized by MPO and EPO, respectively, for generating the $^{\bullet}$OH DNA adduct (Figure 3). Moreover, the requirement for peroxidase-generated hypohalous acid and $O_2^{\bullet-}$ in DNA modification by leukocytes activated in the presence of transition metal ion chelators was apparent by demonstrating that 8-OHG formation by neutrophils and eosinophils was attenuated in the presence of peroxidase inhibitors (e.g., aminotriazole, cyanide), hypohalous acid scavengers (e.g., methionine), and catalytically active SOD. The inhibitory activity of catalytically active catalase is attributable to its ability to block hypohalous acid formation by the peroxidase−H_2O_2−halide system of leukocytes. Finally, it should be noted that all of the reactions in the present study were performed in the presence of the chelator DTPA and used buffers, reagents, and enzymes which were chelex-treated to avoid any potential adventitious transition metal ion-catalyzed formation of $^{\bullet}$OH. Taken together, these results demonstrate that one potential mechanism for generating $^{\bullet}$OH and promoting nucleotide and DNA damage is through reaction of $O_2^{\bullet-}$ with leukocyte-generated hypohalous acids.

Substantial evidence exists linking inflammation, phagocyte-generated oxidants, and peroxidases to carcinogenesis. Clinical studies have documented the association between inflammation and cancer for decades (*1*−*7, 70, 71*). However, the reaction mechanisms responsible for the association are not established. Stimulated neutrophils are capable of inducing genotoxic effects, such as DNA strand breaks (*32, 72*), sister chromatid exchanges (*73*) and mutation (*74, 75*), and promotion of neoplastic transformation in nearby cells (*74, 76*). MPO-generated reactive chlorinating species have been shown to be mutagenic to bacteria (*77*), and MPO has been indirectly implicated in playing a role in carcinogenesis through both activation of procarcinogens to genotoxic intermediates and the potentiation of xenobiotic carcinogenicity (*78, 79*). Recent genetic studies implicate MPO in the

development of lung cancer. Individuals possessing a relatively abundant polymorphism associated with diminished MPO expression demonstrated a dramatic reduction in the relative risk for development of nonsmall cell lung cancers (*80*). The results of the present study suggest that a potential mechanism accounting for some of these observations may be through MPO-dependent damage of DNA by •OH.

Similarly, although a potential role for eosinophils in promoting DNA oxidative damage, carcinogenesis, or metastatic conversion of a malignancy has not yet been explored, the idea is not without precedent. Multiple chronic parasitic infections are causally linked to subsequent development of cancer and represent a leading cause of cancer mortality in some underdeveloped countries (e.g., *Schistosoma haematobium* and bladder cancer, or *Opisthorcis viverrini* and cholangiocarcinoma) (*21, 81*−*84*). Eosinophilic infiltration is a hallmark of these disorders. Interestingly, recent studies demonstrated that the vast majority of breast cancer biopsies contain eosinophils and intensely stain for EPO (*85*). Significant increases in the content of •OH-generated lesions in the DNA from cancerous and metastatic breast tissues have been noted (*12, 13*). Studies of hormone-responsive tissues such as the breast and uterus suggest that eosinophil migration occurs in response to estrogen administration (*86*), and it has long been recognized that elevated peroxidase activity is present in human breast cancers (*87, 88*). Moreover, eosinophil infiltration in lymphomas and malignancies on cutaneous or mucosal surfaces are common (*29, 30*). The function of eosinophils in cancer is uncertain. Although it is assumed that they play a protective role in tissue surveillance, the results of the present study raise the interesting possibility that they may also potentially contribute to DNA oxidative damage, cancer development, or metastases formation.

Previous studies examining DNA damage by activated neutrophils have primarily focused on their ability to promote DNA strand breaks in neighboring cells (*32, 33, 36, 37*). DNA damage in these studies is typically blocked by catalase and significantly increased by addition of Fe^{2+} salts into the media (*35, 36, 89*). The results of the present study suggest that halide-dependent •OH formation is a free metal ion-independent mechanism that may contribute to DNA damage observed in these models. Consistent with this suggestion, several investigators have observed that repair of DNA strand breaks induced by stimulated neutrophils is slower than the repair rate of similar levels of DNA damage induced by nonphagocytic sources of H_2O_2 (*72, 89*). Furthermore, although HOCl does not directly generate DNA strand breaks in human lymphocytes, activation of neutrophils in the presence of MPO inhibitors is reported to significantly decrease the extent of DNA strand breaks, as well as increase the rate of repair (*72*). Thus, in addition to their potential role in damaging DNA and free nucleobases through •OH formation, peroxidase-generated hypohalous acids might also potentially contribute to increased risk for cancer development by inhibiting DNA repair mechanisms at the sites of oxidant production.

One striking feature of the cytotoxicity studies was the apparent ability of injured cells to undergo repair and ultimately become mitotically active, as assessed by clonogenic survival assays (Table 1). For example, although almost 40% of preloaded [^{14}C]adenine was released from intracellular pools of HA1 cells exposed to both hyperoxia and the HOCl-generating system, less than 20% of these cells ultimately died (i.e., became clonogenically incompetent, Table 1). These results suggest that at sites of chronic inflammation, a "gray zone" will likely exist where DNA, RNA, and free nucleotide damage occurs, but which is subsequently processed and repaired. The chronic nature of many inflammatory conditions associated with enhanced risk for cancer development (e.g., inflammatory bowel diseases, hepatitis, numerous parasitic infections) often spans decades. Thus, even a modest lack of fidelity in repair mechanisms could potentially result in mutations at critical sites which ultimately lead to malignant transformation and cancer development.

One key question was whether •OH generated by interaction of $O_2^{\bullet-}$ and hypohalous acid could damage cellular DNA. The results of the present study demonstrate that DNA, RNA, and free cytosolic nucleotides are all potential targets for •OH-mediated damage in cells exposed to an exogenous hypohalous acid-generating system, particularly under conditions of enhanced intracellular $O_2^{\bullet-}$ production (Table 1). Mitochondrial DNA, and to a lesser extent RNA and cytosolic nucleobases, demonstrated the greatest levels of 8-OHG following exposure of cells to the MPO−H_2O_2−Cl^- system. A likely explanation for the enhanced susceptibility of mitochondrial DNA to oxidative damage is that mitochondria serve as a primary source of intracellular $O_2^{\bullet-}$ production. This would facilitate •OH formation in close proximity to mitochondrial DNA. In addition, mitochondrial DNA does not bear a protective coat of highly basic histone proteins; thus, protection from reactive oxidants such as hypohalous acids is far less than that observed in the nucleus. Finally, DNA repair mechanisms in mitochondria are less efficient than those in the nucleus (*68*).

It is also interesting that 8-OHG was formed in the cytosolic pool of cells exposed to the peroxidase−H_2O_2−halide system of leukocytes (Table 1). Oxidized nucleobases from cellular pools may be incorporated into DNA, leading to substitution mutations in the case of 8-OHG (*14, 15*). Although there are DNA repair systems which recognize 8-OHG with high affinity, the fidelity of these repair enzymes is not absolute, and increased cancer risk with accompanying increased levels of oxidative DNA damage is known to occur when defects in the DNA repair systems are present (*90, 91*).

Few studies have reported determination of 8-OHG in RNA. The highly reactive nature of •OH, and the subsequent limited diffusional distance of the species before productive encounter, suggests that much of the •OH generated in cells exposed to HOCl during hyperoxia also occurred in the cytosol. Redox cycling of cytosolic flavoproteins may serve as a source of $O_2^{\bullet-}$ under these conditions (*69*). Because of the very rapid removal of 8-OHG from DNA, but not RNA (*92*), quantification of 8-OHG in RNA might represent a useful complimentary method for evaluating the intracellular source of cellular oxidative damage induced in cells. Whether oxidative modification of RNA plays a role in transcriptional regulation at sites of inflammation remains to be determined.

In summary, the present study identifies a novel mechanism for 8-OHG formation by activated phagocytic cells. The role of peroxidase-generated oxidants in contributing to the etiology of inflammation-related cancers is unknown.

We focused on the formation of 8-OHG as a marker of •OH formation and DNA damage because this mutagenic base both is implicated in cancer development and is easily quantified by sensitive and specific methods. Although 8-OHG represents one of the more abundant •OH-mediated products formed during DNA oxidation, numerous additional types of base modifications occur as a result of DNA exposure to •OH. Indeed, we observed multiple additional peaks of unknown identity during HPLC-EC analyses, and even more electrochemically silent oxidation products of DNA are undoubtedly formed. It is tempting to speculate that hypohalous acids might act directly on DNA, RNA, or cytosolic nucleobases, generating halogenated oxidation products. Future studies in this area are warranted since these might serve as powerful tools to identify a direct role for MPO and EPO in promoting oxidative damage of nucleotides in vivo.

ACKNOWLEDGMENT

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

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BI992809Y