Hypochlorous Acid Produced by the Myeloperoxidase System of Human Phagocytes Induces Covalent Cross-Links between DNA and Protein†

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ABSTRACT: Phagocytic oxidants have been implicated in tissue injury and oncogenesis, and their pathophysiological role in modifying nucleobases and amino acids has been widely explored. Their ability to cross-link proteins and DNA, however, has not been considered, even though reversible DNA−protein interactions are key to gene expression and to DNA replication and repair. In the current studies, we show that hypochlorous acid (HOCl), generated by the myeloperoxidase−hydrogen peroxide−chloride system of phagocytes, cross-links single-stranded DNA-binding protein (SSB) to single-stranded oligonucleotides. Exposure of SSB and a homopolymer of radiolabeled thymidine (dT_{40}) to HOCl resulted in the formation of a radiolabeled band with slower mobility than the free oligonucleotide, as determined by denaturing polyacrylamide gel electrophoresis. This radiolabeled band did not appear if the reaction mixture was treated with protease or nuclease, indicating that it represents a covalent complex of DNA and protein. Oligonucleotides of adenosine and cytidine behaved similarly to the thymidine oligonucleotide, demonstrating that they are also capable of participating in the cross-linking reaction. The covalent complex of radiolabeled dT_{40} and SSB was also generated by chloramines and the complete myeloperoxidase−hydrogen peroxide−chloride system. The enzymatic reaction required each component of the system and was inhibited by heme poisons and chloride-free conditions, implicating myeloperoxidase and HOCl. DNA−protein cross-links were generated in *Escherichia coli* exposed to HOCl, suggesting that double-stranded DNA is also a target for the reaction. These results indicate that long-lived chloramines and HOCl generated by myeloperoxidase can generate covalent DNA−protein cross-links that may contribute to the mutagenic and cytotoxic effects of phagocytes on microbial pathogens and host tissue.

Chronic inflammation is one important risk factor for oncogenesis, and phagocytic white blood cells play a leading role in the inflammatory response. These cells are a well-characterized source of reactive intermediates, raising the possibility that the oxidants they secrete promote tumor formation in vivo (*1*−*3*).

In vitro studies have shown that phagocytic oxidants can break DNA strands (*4*−*7*), mutate bacterial DNA (*8*−*11*), cause cytogenetic changes in mammalian cells (*12*−*14*), and promote malignant transformation (*15*). One mechanism may involve direct damage to nucleic acids. For example, DNA bases are hydroxylated by hydroxyl radical and singlet oxygen (*16*, *17*), nitrated by reactive nitrogen species (*18*−*20*), chlorinated and brominated by hypohalous acid (*21*−*24*), and modified by reactive carbonyls that result from lipid peroxidation (*25*). These pathways may be biologically relevant because oxidized nucleic acids have been detected in DNA extracted from cells exposed to oxidizing conditions (*25*, *26*). They also have been found in human urine (*27*). The mechanisms that oxidize nucleobases in vivo, however, and their relevance to human pathology are incompletely understood.

Noncovalent interactions between DNA and proteins are essential for proper cellular function. The potential role of DNA−protein cross-linking in mutagenesis and tissue injury, however, has received surprisingly little attention. Ionizing radiation and UV radiation promote malignant transformation in vitro and in vivo (*1*−*3*), and both of these toxic insults promote DNA−protein cross-linking in model systems and cultured cells (*28*−*33*), possibly through the formation of thymine−tyrosine adducts (*34*−*36*). The cellular consequences of DNA−protein cross-links are poorly understood. Many DNA repair systems work by recognizing either DNA strand breaks or large bulky lesions that disrupt the integrity of the DNA double helix (*37*−*40*). If a covalent linkage can form between a DNA-binding protein and its target DNA without grossly disrupting normal DNA−protein interactions, a cell may not recognize and effectively repair this damage. Therefore, DNA−protein cross-linking could be harmful to a cell.

The heme enzyme myeloperoxidase is one agent that appears to be capable of oxidizing DNA or cross-linking it to proteins. Activated neutrophils and monocytes secrete large quantities of this enzyme into the phagolysosome and extracellular environment (*41*−*43*). Myeloperoxidase uses hydrogen peroxide (H_2O_2) generated by activated phagocytes

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to catalyze the formation of hypochlorous acid (HOCl) from chloride ion (Cl^-) (*44*).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \quad (1)$$

HOCl is an important component of host defenses against invading organisms. A potent oxidant, it modifies proteins, lipids, and nucleic acids (*21*−*24*, *45*−*48*). Because of its reactivity and ability to cross lipid membranes, it also has the potential to damage host tissues. Oxidants are thought to contribute to the pathogenesis of diseases, ranging from arthritis to ischemia−reperfusion injury and inflammatory bowel disease (*1*, *49*, *50*). Chlorinated proteins have been detected in atherosclerotic tissue (*51*, *52*), implicating myeloperoxidase as one agent for oxidative damage in humans.

HOCl cross-links proteins in vitro (*53*, *54*), raising the possibility that it might similarly damage other biomolecules. In the current studies, we show that HOCl, generated by the myeloperoxidase−H_2O_2−Cl^- system, generates covalent cross-links between DNA and protein. This raises the possibility that oxidizing intermediates generated by myeloperoxidase modify polymeric nucleic acids in host cells at sites of inflammation, setting the scene for carcinogenesis, cytotoxicity, or other deleterious changes.

EXPERIMENTAL PROCEDURES

Materials. Single-stranded DNA binding protein (SSB) and T4 polynucleotide kinase were obtained from Promega Corp. (Madison, WI). DNAse I, catalase (thymol free, from beef liver), and S1 nuclease were obtained from Boehringer Mannheim (Indianapolis, IN). *Escherichia coli* (ATCC 11775) was provided by Dr. Henry Rosen (University of Washington, Seattle, WA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA), unless otherwise indicated.

Isolation of Myeloperoxidase. Myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was extracted with cetyltrimethylammonium bromide from human leukocytes obtained by leukopheresis. Solubilized myeloperoxidase was purified by lectin affinity chromatography and size-exclusion chromatography, as previously described (*55*, *56*). The enzyme (A_{430}/A_{280} ratio of 0.6) was dialyzed against distilled water and stored in 50% glycerol solution at −20 °C. Enzyme concentration was determined spectrophotometrically ($\epsilon = 178$ mM^{-1} cm^{-1} (*57*)).

Preparation of DNA Oligonucleotides and SSB Protein. DNA oligonucleotides (Integrated DNA Technologies, Ames, IA) were purified by polyacrylamide gel electrophoresis (15% acrylamide, 8 M urea, 0.1% SDS), as described previously (*58*). Concentrations were determined spectrophotometrically using the relationship $A_{260} = 1$ of single-stranded DNA = 33 μg/mL (*59*). Oligonucleotides were end-labeled, using ^{32}P-γ-ATP (NEN Life Sciences; Boston, MA) and T4 polynucleotide kinase (*60*), and separated from free radiolabeled nucleotides by size-exclusion chromatography, using a column (Bio-Rad Micro Bio-Spin 6 chromatography column; Bio-Rad, Hercules, CA) that had been equilibrated with buffer A (see below). SSB protein was dialyzed against buffer A and stored in 50% glycerol at −20 °C. Protein concentration was determined spectrophotometrically ($\epsilon_{280} = 1.13 \times 10^5$ M^{-1} cm^{-1} for SSB tetramer (*61*)).

Reaction Conditions for SSB−Oligonucleotide Cross-Linking. For cross-linking by reagent HOCl, 1 pmol each of SSB tetramer and DNA oligonucleotide were incubated in a final volume of 10 μL at 25 °C in buffer A (20 mM sodium phosphate (pH 7.4), 100 mM NaCl, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)). Reactions were initiated by adding HOCl, incubated for the indicated time, and terminated by adding reducing buffer (3.3% β-mercaptoethanol, 160 mM Tris-base (pH 6.8), 3.3% SDS, 33% glycerol, 0.004% bromophenol blue) and heating at 100 °C for 2 min. Reactions with chloramine were performed at 37 °C in buffer A. Chloramines were formed by incubating 10 mM taurine with 1 mM HOCl for 30 min. Chloramine formation was confirmed by monitoring absorbance at 252 nm (*62*). Reactions were initiated by chloramine addition, incubated for 60 min, and terminated by adding reducing buffer. For cross-linking by myeloperoxidase, the SSB tetramer and the DNA oligonucleotide were incubated at 37 °C in buffer A supplemented with 10 nM myeloperoxidase. Reactions were initiated by adding H_2O_2, incubated for the indicated time, and terminated by adding reducing buffer and heating at 100 °C for 2 min. For Cl^--free reactions, NaCl was omitted from buffer A. DTPA was included in the reaction mixture to chelate free metal ions that could potentially generate reactive oxygen species (*63*).

Denaturing Polyacrylamide Gel Electrophoretic Analysis of DNA−Protein Complexes. Following the addition of an equal volume of reducing buffer and heating at 100 °C for 2 min, reaction mixtures were subjected to electrophoresis on a 12% polyacrylamide gel containing 0.1% SDS in 375 mM Tris-base, pH 8.8. The stacking gel was made with 5% polyacrylamide and contained 0.1% SDS in 125 mM Tris-base, pH 6.8. The electrophoresis buffer consisted of 25 mM Tris-base, 250 mM glycine, and 1% SDS. Polyacrylamide gels were dried under vacuum and subjected to autoradiography (X-OMAT LS film; Eastman Kodak Co., Rochester, NY).

SDS−KCl Precipitation of DNA−Protein Cross-Links Isolated from Bacteria. Cultures of *E. coli* (ATCC 11775) were grown in Luria broth at 37 °C to a final concentration of 1×10^9 cells mL^{-1}, as determined spectrophotometrically by measuring absorbance at λ_{600}. Cultures were separated into 1 mL aliquots and pelleted by centrifugation, the supernatant was removed, and the pellets were rinsed twice in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4). Pellets were resuspended in 50 μL of PBS. Increasing concentrations of HOCl (between 0 and 1000 μM) were added in 50 μL of PBS for a final reaction volume of 100 μL. SDS−KCl precipitation reactions were performed as previously described (*64*). Briefly, cells were lysed with 2% SDS, 1 mM PMSF, and 20 mM Tris-HCl, pH 7.5. DNA was sheared by passing the cell lysates through a 21-gauge needle, taking care to avoid extensive foaming. SDS−protein complexes were formed by adding 100 mM KCl in 20 mM Tris-HCl (pH 7.5), incubating the mixture for 10 min at 65 °C, and then placing it on ice for 10 min. Complexes were pelleted by centrifugation (10,000*g* for 5 min). The pelleted complexes were resuspended in KCl−Tris buffer and pelleted by centrifugation two more times. The isolated complexes were then treated with 0.2 mg/mL proteinase K in 100 mM KCl, 20 mM Tris-HCl (pH 7.5), and 10 mM EDTA at

50 °C for 3 h. After digestion, the samples were treated with 50 μL of a 4 μg/mL solution of BSA[1] and placed on ice. The samples were pelleted by centrifugation, and the supernatants were treated with freshly prepared Hoescht reagent 33258 (250 ng/mL). Fluorescence was measured by excitation at 360 nm and emission at 460 nm.

Other Procedures. Radiolabeled materials were quantified by phosphor imaging (Storm PhosphorImager analysis; Molecular Dynamics Inc., Sunnyvale, CA). UV absorption spectra were obtained at room temperature using a scanning UV−visible spectrophotometer (Cary 100 Bio; Varian, Sugar Land, TX). Concentrations of H_2O_2 and HOCl were determined spectrophotometrically using $\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$ and $\epsilon_{292} = 350\ M^{-1}\ cm^{-1}$, respectively (*65, 66*). Unless otherwise indicated, data represent the results from at least three independent experiments.

RESULTS

Hypochlorous Acid Covalently Cross-Links SSB Protein to Single-Stranded Deoxynucleotide Homopolymers. To determine whether oxidants generated by myeloperoxidase promote the cross-linking of DNA and protein, we investigated a simple model system consisting of single-stranded DNA binding protein (SSB) and oligomers of either deoxythymidine, deoxyadenosine, or deoxycytidine. Deoxyguanosine oligomers were not used because they are insoluble under our reaction conditions. SSB is an abundant bacterial protein involved in DNA replication, recombination, and repair (*61*). Several homologues exist in both yeast and humans. SSB binds single-stranded DNA in a sequence-independent manner with extremely high affinity (*61, 67*). Importantly, it also binds short homopolymers of DNA with high affinity. Homopolymers were used in our model system to determine which of the nucleotides can cross-link to protein.

Because HOCl is the major product of the myeloperoxidase−H_2O_2−Cl^- system, we first determined whether this oxidant can cross-link protein to DNA. Reactions were performed at neutral pH in phosphate buffer containing plasma concentrations of Cl^-. DTPA was included in the reaction mixture to chelate free metal ions that might catalyze cross-linking reactions. DNA and protein were preincubated before the addition of the oxidant. Under these conditions, SSB and oligomer form a complex of four SSB molecules and one DNA molecule (*68*). We exposed this noncovalent complex to 200 μM HOCl for 30 min and terminated the reaction by adding reducing buffer. The reaction mixture then was analyzed using denaturing polyacrylamide gel electrophoresis and autoradiography. As shown in Figure 1, ^{32}P-labeled thymidine homopolymer (dT_{40}), incubated with SSB protein and then exposed to HOCl, generated one major band of radiolabeled material whose mobility was slower than that of the oligonucleotide. Formation of this material required DNA, protein, and oxidant; no complex was observed with DNA and HOCl alone (Figure 1, lane 7). This requirement for both protein and DNA, and the ability of the complex to withstand denaturing conditions, suggest that HOCl covalently cross-links protein and DNA.

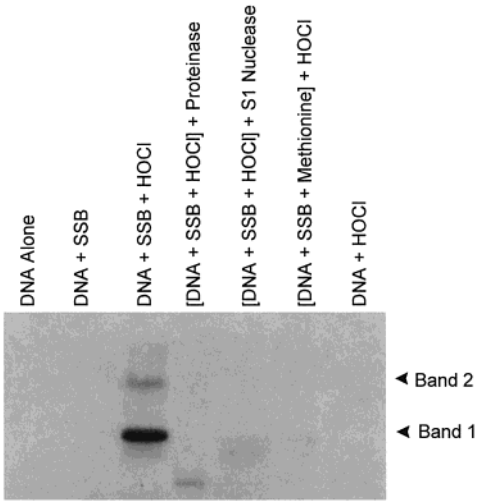

FIGURE 1: Denaturing polyacrylamide gel electrophoresis and autoradiographic analysis of radiolabeled dT_{40} and SSB exposed to HOCl. SSB was incubated with end-labeled [^{32}P]dT_{40} oligomer in buffer A (20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, 0.1 mM DTPA) for 20 min at 25 °C. The reaction was initiated by the addition of 200 μM HOCl, and the reaction mixture was incubated for 30 min at 37 °C. The reaction was terminated by adding reducing buffer (final concentrations of 1.6% β-mercaptoethanol and 1.6% SDS) and then boiling the mixture for 2 min. The reaction mixture was analyzed using denaturing (0.1% SDS) polyacrylamide gel electrophoresis and autoradiography. Where indicated, individual components of the reaction system were omitted, the complete reaction mixture (following the 30 min incubation at 37 °C) was treated with proteinase K (0.5 mg) or S1 nuclease (20 U) and incubated for an additional 60 min at 37 °C prior to analysis, or methionine (5 mM) was added to the reaction mixture prior to HOCl. Free radiolabeled oligomer, which ran at the bromophenol blue dye front, is not shown.

To confirm that the radiolabled material with altered electrophoretic mobility contained both protein and DNA, we first incubated radiolabeled dT_{40} oligomer and SSB with HOCl, and then exposed the resulting complex to S1 nuclease or proteinase K. The complex was destroyed by either enzyme (Figure 1, lanes 4 and 5), indicating that it had contained both protein and DNA. When methionine, a known scavenger of HOCl, was added to the reaction mixture before the HOCl was added, no complex formed (Figure 1, lane 6). This observation suggests that HOCl or a reactive intermediate derived from HOCl mediates the reaction. HOCl also promoted DNA−protein cross-linking when the dT_{40} homopolymer was replaced with a 40-mer homopolymer of either cytidine or adenosine (data not shown). Collectively, these results indicate that HOCl generates covalent DNA−protein cross-links and suggest that thymine, adenine, and cytosine can participate in the reaction, or that SSB cross-links to deoxyribose (*69*).

Hypochlorous Acid-Induced Cross-Linking of Protein and DNA is Concentration-Dependent and Time-Dependent. Raising the concentration of HOCl in the reaction mixture increased the formation of dT_{40}−SSB complexes (Figure 2). At low concentrations of oxidant ($\leq$200 μM), one major band of material (band 1) was observed. A second major band (band 2) became apparent at higher HOCl concentrations (Figure 2A). The multiple bands may reflect complexes with varying numbers of SSB monomers and/or dT_{40} oligomers.

[1] Abbreviations: BSA, bovine serum albumin; dA_{40}, deoxyadenosine 40-mer; dC_{40}, deoxycytidine 40-mer; dT_{40}, deoxythymidine 40-mer; DTPA, diethylenetriaminepentaacetic acid; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; KCl, potassium chloride; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SSB, single-stranded DNA binding protein.

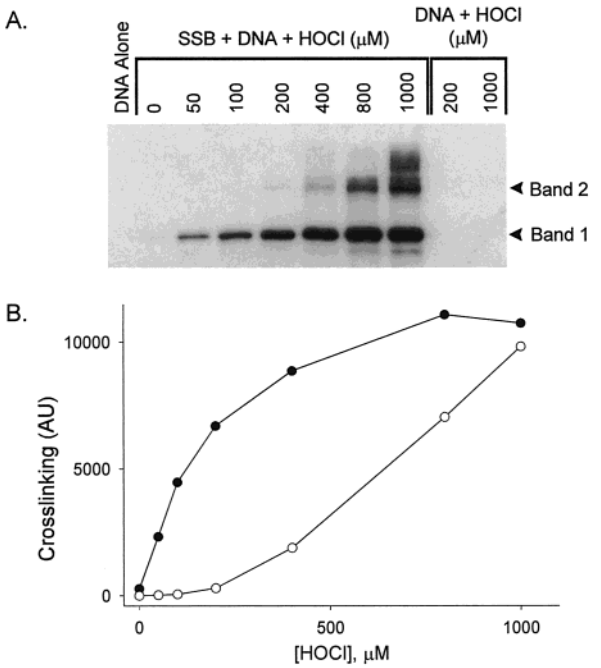

FIGURE 2: Concentration dependence of DNA−protein cross-linking by HOCl. SSB and [^{32}P]dT$_{40}$ oligomer were exposed to the indicated concentration of HOCl as described in the legend to Figure 1, and then incubated for 30 min at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (A), and the amount of radiolabeled material in band 1 and band 2 was quantified by phosphor imaging (B).

Alternatively, the complex formed at low oxidant concentrations may have a different conformation than that formed at high concentrations and, therefore, may have a different apparent mobility on polyacrylamide gel. No material with altered mobility was observed when DNA alone was incubated with 200 or 1000 μM HOCl, indicating that interstrand or intrastrand cross-linking of DNA did not produce the complex.

Adding 200 μM HOCl to radiolabeled dT$_{40}$ and SSB increased the formation of the protein−DNA complex in a time-dependent manner (Figure 3). Production of band 1 increased rapidly over the first 50 min and gradually over the next 200 min (Figure 3B). In contrast, the material in band 2 was initially absent under these reaction conditions and then gradually accumulated. Collectively, these observations raise the possibility that band 1 is an intermediate in the reaction pathway to the formation of band 2.

To determine whether lower concentrations of oxidant would promote complex formation, we examined the progress curve that resulted when radiolabled dT$_{40}$ and SSB were exposed to 100 nM HOCl. As shown in Figure 4, complex was seen as early as in 4 h, even at this very low concentration of oxidant, and cross-linking continued throughout the 24 h incubation.

The Order of Reactants Affects DNA−Protein Cross-Linking. To gain insight into the chemistry behind the cross-linking reaction, we varied the order of addition of the reactants. Our standard procedure was to preincubate the DNA and protein and then to expose the noncovalent DNA−SSB complex to HOCl. If we added HOCl to the DNA before we added the protein, the covalently cross-linked complex also formed (data not shown). The relative yield and apparent

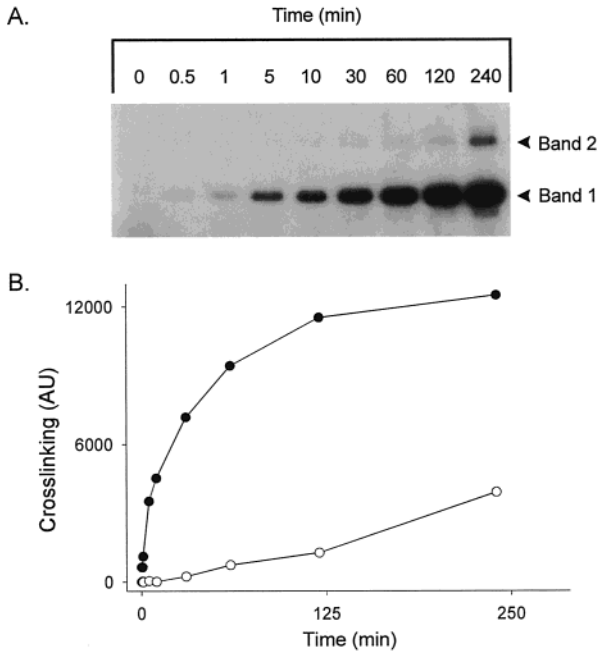

FIGURE 3: Time dependence of DNA−protein cross-linking by HOCl. SSB and [^{32}P]dT$_{40}$ oligomer were exposed to 200 μM HOCl as described in the legend to Figure 1, and then incubated for the indicated time at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (A), and the amount of radiolabeled material in band 1 and band 2 was quantified by phosphor imaging (B).

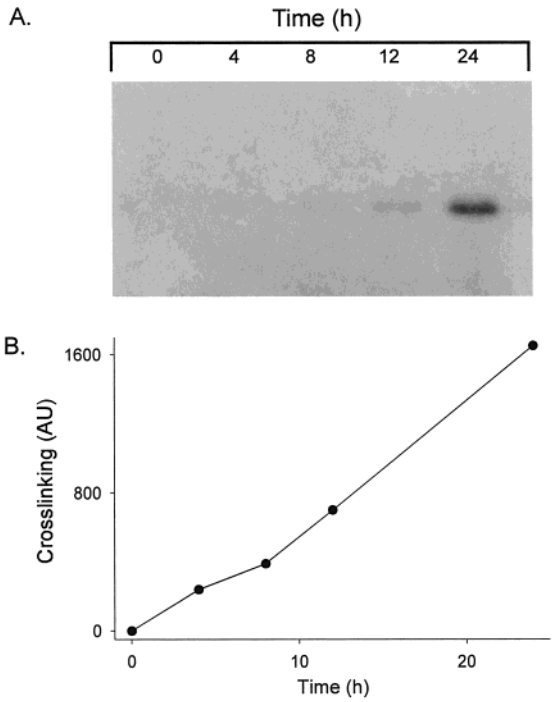

FIGURE 4: Time dependence of DNA−protein cross-linking by low concentrations of HOCl. SSB and [^{32}P]dT$_{40}$ oligomer were exposed to 100 nM HOCl as described in the legend to Figure 1, and then incubated for the indicated time at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (A), and the amount of radiolabeled material was quantified by phosphor imaging (B).

electrophoretic mobility of the radiolabeled material were similar to those found when standard reaction conditions

were used. In contrast, the complex failed to appear when we added HOCl to SSB and subsequently added radiolabeled dT_{40}. We obtained similar results when the dT_{40} oligomer was replaced with either a dC_{40} or a dA_{40} oligomer. Like the progress curve of the reaction, these observations suggest that relatively long-lived intermediates, derived from the interaction of HOCl and oligonucleotide, play an important role in the cross-linking reaction.

Hypochlorous Acid Induces SSB Cross-Linking. Failure to obtain a complex when we added DNA to the reaction mixture after the addition of SSB and HOCl suggests that the reactive moieties on the protein (such as thiol groups) scavenge oxidant. Alternatively, HOCl might oxidize SSB to a form that is unable to bind to DNA. Indeed, HOCl is known to chlorinate amino and aromatic groups and to cross-link proteins (*52*−*54*, *62*, *69*, *70*). To determine whether SSB cross-linking might account in part for the absence of complex, we exposed the protein to HOCl concentrations ranging from 0.1 to 1 mM and then analyzed the reaction mixture by SDS−PAGE and Coomassie blue staining. In the absence of HOCl, the protein migrated as a single band with an apparent molecular mass of ∼19 kDa, the expected molecular mass of the monomeric protein. SSB exposed to HOCl concentrations of less than 1 mM demonstrated a second prominent band that migrated with an apparent molecular mass of ∼40 kDa, the approximate molecular masst for a dimer of SSB protein (data not shown). The yield of the 40 kDa species was proportional to the concentration of HOCl in the reaction mixture.

When SSB was replaced with an equimolar amount of bovine serum albumin (BSA) and the reaction mixture was mixed with <1 mM HOCl, a single band of material appeared on SDS−PAGE. It migrated with the same apparent molecular mass as the native protein. At high concentrations of HOCl (≥1 mM), both SSB and BSA were undetectable, suggesting that the proteins had fragmented into low molecular weight peptides or had lost the ability to react with Coomassie blue. Because SSB—but not albumin—is known to form a tetrameric complex under these experimental conditions, these observations suggest that protein−protein cross-linking by HOCl is promoted by intimate contact of the proteins. They also suggest that cross-linking of SSB to itself accounts in part for the failure of HOCl-oxidized SSB to participate in complex formation.

BSA Fails To Inhibit HOCl's Ability To Cross-Link DNA to SSB. To establish whether the presence of a protein that does not bind DNA can inhibit DNA−protein cross-linking by HOCl, we included increasing concentrations of BSA in the SSB−dT_{40} reaction mixture. BSA was added either immediately prior to the HOCl addition (Figure 5B) or during the preincubation of SSB with DNA (Figure 5C). BSA had little effect on complex formation, as assessed by denaturing polyacrylamide gel electrophoresis and autoradiography, even when its molar concentration was 100 times that of SSB (Figure 5B and C, lanes 3).

To determine whether HOCl can cross-link DNA to BSA in the absence of SSB, we exposed ^{32}P-labeled deoxynucleotide homopolymer (either dT, dA, or dC) and BSA to increasing HOCl concentrations for 30 min. No complex was detectable in a reaction mixture subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (data not shown). This observation indicates that BSA was unable

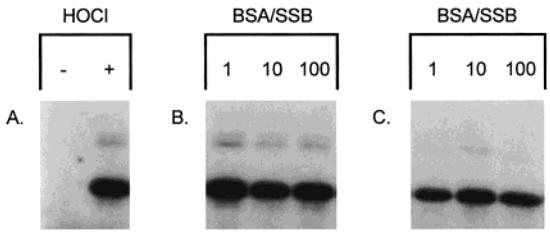

FIGURE 5: Effect of BSA on DNA−protein cross-linking by HOCl. SSB and [^{32}P]dT_{40} oligomer reaction mixture was supplemented with the indicated concentration of BSA, exposed to 200 μM HOCl as described in the legend to Figure 1, and then incubated for 20 min at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography. (A) DNA−protein cross-linking in the absence of BSA. SSB and [^{32}P]dT_{40} were incubated with (+) and without (−) HOCl and subjected to analysis. (B) DNA−protein cross-linking in the presence of BSA. SSB and [^{32}P]dT_{40} were incubated for 20 min, and the indicated ratio of BSA (BSA/SSB, mol/mol) was added followed by the addition of HOCl and analysis. (C) DNA−protein cross-linking in the presence of BSA. SSB [^{32}P]dT_{40} and the indicated amount of BSA (BSA/SSB, mol/mol) were incubated for 20 min, followed by the addition of HOCl and analysis.

to covalently cross-link to DNA in the presence of HOCl under our experimental conditions, suggesting that complex formation requires close interaction of protein and DNA. Alternatively, the failure to observe cross-linking with BSA may reflect differences between this protein and SSB in the accessibility of groups susceptible to oxidative modification or other reactions.

Chloramines Induce DNA−Protein Cross-Linking. The progress curve of the cross-linking reaction suggested that long-lived intermediates derived from HOCl are responsible for cross-linking the protein and DNA. A potential intermediate involves chloramines, which result from the oxidation of primary amines:

$$HOCl + R{-}NH_2 \rightarrow R{-}NHCl + H_2O \qquad (2)$$

Chloramines are relatively stable compounds that exist in equilibrium with primary amines (*62*, *71*).

Complex formation was observed when taurine chloramine (1:10 mol ratio of HOCl to taurine) was added to radiolabeled dT_{40} and SSB (Figure 6). The level of cross-linking seen for taurine chloramine was similar to that observed with an equimolar amount of reagent HOCl. These observations indicate that chloramines also are able to promote complex formation. The extent of DNA−protein cross-linking increased with increasing concentrations of chloramine. In contrast, high concentrations of HOCl (1 mM) resulted in an apparent decrease in DNA−protein cross-linking (Figure 6). As noted above, under these conditions, SSB was undetectable on denaturing polyacrylamide gel electrophoresis, suggesting that the protein had fragmented into low molecular weight peptides or had been oxidized to a form that was unable to undergo the cross-linking reaction.

The Myeloperoxidase−H_2O_2−Cl^- System Cross-Links DNA to SSB in a Reaction That Requires HOCl. To investigate the potential physiological relevance of HOCl as a DNA−protein cross-linking reagent, we determined whether the complete myeloperoxidase−H_2O_2−Cl^- system can induce complex formation. After we exposed radiolabeled dT_{40} and SSB to 10 nM myeloperoxidase, in the presence of 200

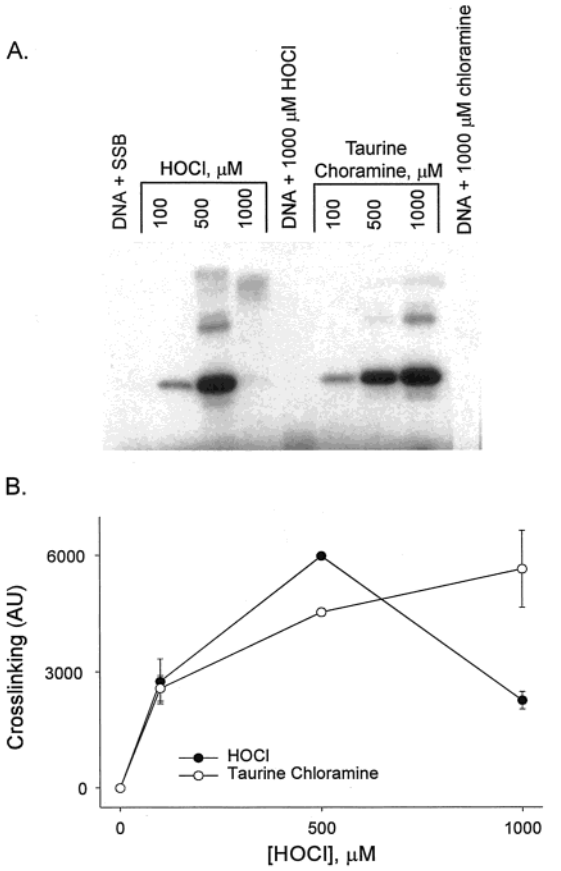

FIGURE 6: Cross-linking of DNA and protein by chloramines. SSB and [^{32}P]dT_{40} oligomer were exposed to either HOCl or taurine chloramine (1:10 mol ratio of HOCl to taurine) as described in the legend to Figure 1, and then incubated for 60 min at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (A), and the amount of radiolabeled material was quantified by phosphor imaging (B). Results represent the mean and the standard deviation of two experiments, with duplicate determinations for experiment.

μM H_2O_2 and 100 mM Cl^-, material with altered electrophoretic mobility was detectable by denaturing polyacrylamide electrophoresis and autoradiography (Figure 7, lane 5). The mobility of the radiolabeled material was identical to that observed when the enzymatic system was replaced with 200 μM HOCl (Figure 7, lane 4). The myeloperoxidase−H_2O_2−Cl^- system also promoted DNA−protein cross-linking when the dT_{40} homopolymer was replaced with a 40-mer homopolymer of either cytidine or adenosine (data not shown).

Formation of the complex required enzyme, peroxide, and halide (Figure 7, lanes 6−8); if SSB was omitted from the reaction mixture, no slowly migrating radiolabeled material was observed. The reaction was inhibited by the peroxide scavenger catalase and the heme poisons, azide and cyanide, implicating H_2O_2 and myeloperoxidase in the reaction. Production of the complex exhibited a lag phase of ~10 min; production then rapidly increased over the next 125 min (Figure 8). Collectively, these results indicate that HOCl generated by myeloperoxidase generates a covalent complex between DNA and protein. The lag phase of the myeloperoxidase-dependent reaction suggests that relatively long-lived reactive intermediates derived from HOCl are involved in forming the DNA−protein cross-link.

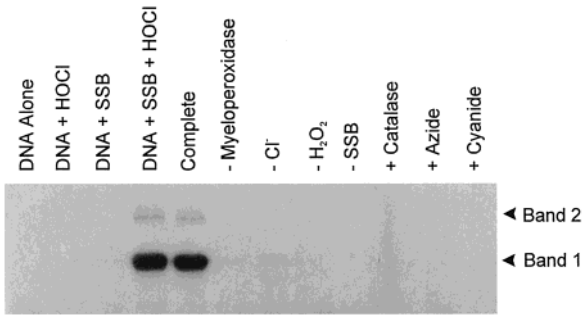

FIGURE 7: Cross-linking of DNA and protein by the myeloperoxidase−H_2O_2−Cl^- system. SSB was incubated with end-labeled [^{32}P]dT_{40} oligomer in buffer A (20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, 0.1 mM DTPA) supplemented with 10 nM myeloperoxidase for 20 min at 25 °C. The reaction was initiated by addition of 200 μM H_2O_2, and the reaction mixture was incubated for 60 min at 37 °C. The reaction was terminated by addition of reducing buffer and boiling for 2 min. The reaction mixture was analyzed using denaturing polyacrylamide gel electrophoresis and autoradiography. Where indicated, 200 μM HOCl was substituted for the enzymatic system, individual components of the enzymatic reaction system were omitted, or catalase (400 nM), azide (20 mM), or cyanide (20 mM) was present together with the complete enzymatic system.

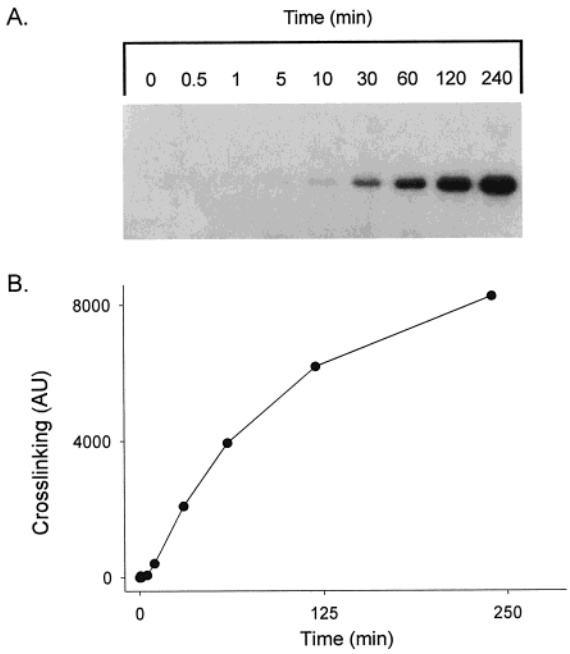

FIGURE 8: Time dependence of DNA−protein cross-linking by the myeloperoxidase−H_2O_2−Cl^- system. SSB and [^{32}P]dT_{40} oligomer was exposed to the complete myeloperoxidase enzymatic system as described in the legend to Figure 6. Following the addition of 200 μM H_2O_2, the reaction mixture was incubated for the indicated period of time at 37 °C. The reaction was terminated by addition of reducing buffer. The reaction mixture was subjected to denaturing polyacrylamide gel electrophoresis and autoradiography (A), and the amount of radiolabeled material was quantified by phosphor imaging (B).

Hypochlorous Acid Induces DNA−Protein Cross-Linking in E. coli. We exposed *E. coli* to HOCl to determine whether DNA−protein cross-links were produced in a cellular system. Under these conditions, double-stranded DNA would be the major target for damage. DNA−protein cross-links were measured using the SDS−KCl precipitation method (*72*). In this analytical method, complexes of SDS and protein are precipitated, leaving free DNA in solution. Any DNA that

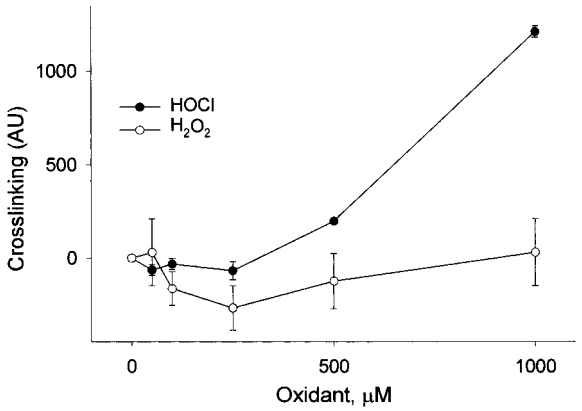

FIGURE 9: DNA−protein cross-link formation in *E. coli*. *E. coli* (1×10^9 cells) was incubated with HOCl in PBS (pH 7.4) for 3 h at 37 °C. Reactions were stopped by the addition of 150 mM methionine, and the cells were immediately lysed with SDS. DNA−protein complexes were precipitated using SDS−KCl. DNA in the protein fraction was quantified by treating with Hoescht reagent 33258 and measuring fluorescence.

is covalently linked to protein, however, will precipitate with the protein. DNA in the protein pellet is then quantified using a fluorescent dye that selectively intercalates into double-stranded DNA (*73*).

Cells were exposed to HOCl or H_2O_2 in a physiological salt solution at neutral pH. After a 3 h incubation at 37 °C, the reaction was stopped with 150 mM methionine, and the DNA that was protein-associated was precipitated and quantified by fluorescence. DNA−protein cross-links were formed in *E. coli* exposed to HOCl (Figure 9). In contrast, H_2O_2 failed to promote the reaction. There was no evidence of DNA−protein cross-linking when the cells were exposed to HOCl concentrations less than 500 μM; these results suggest that other reactive moieties (such as thiols and other reducing agents) were scavenging the oxidant. As with the SSB/oligomer system, DNA−protein cross-linking increased with increasing incubation time (data not shown). These observations indicate that cross-links between double-stranded DNA and proteins are generated in cells exposed to HOCl.

DISCUSSION

Oxidants secreted by phagocytes have been implicated in cytotoxicity at sites of inflammation, host defense mechanisms, and the pathogenesis of cancer (*1*−*3*). Many investigators have explored the oxidative modification of nucleobases, lipids, and amino acids, but the possible role of protein−DNA cross-linking in biological damage has received little attention, even though protein−nucleic acid interactions are critical for proper cellular function.

We have examined the ability of HOCl, generated by myeloperoxidase, to promote cross-linking of DNA and protein both in the SSB protein model system and in *E. coli*. Exposing SSB and the homopolymers of deoxyadenosine, deoxycytidine, or deoxythymidine to HOCl generated a complex that remained intact during electrophoresis under denaturing conditions. The oligonucleotide−SSB complex was undetectable when the reaction mixture was treated with a nuclease or protease, confirming that the reaction generated a covalent cross-link between DNA and the protein. The HOCl-dependent reaction was sensitive to inhibition by methionine, implicating HOCl in the reaction pathway. The DNA−protein complex also formed when we exposed the oligonucleotide and SSB to the complete myeloperoxidase−H_2O_2−Cl^- system. Its production required each component of the reaction mixture and was inhibited by catalase and heme poisons, implicating H_2O_2 and myeloperoxidase. Importantly, myeloperoxidase and H_2O_2 were unable to promote DNA−protein cross-linking in the absence of Cl^-. Despite the high reactivity of HOCl with a wide range of biological substrates, HOCl was also able to induce cross-links between double-stranded DNA and proteins in *E. coli*. Collectively, these results indicate that HOCl, or a reactive intermediate derived from HOCl, covalently cross-links DNA and protein.

HOCl reacts rapidly with primary amines, aromatic compounds, thiols, and unsaturated moieties (*41*−*43*, *45*−*48*, *50*). The time course for DNA−protein cross-linking, however, shows that cross-linking increases over time. The shape of the progress curve suggests that HOCl reacts with DNA or protein to generate a long-lived intermediate that goes on to form a covalent cross-link. The fact that we obtained the complex even if we exposed dT_{40} to HOCl before adding SSB to the reaction mixture is consistent with this possibility. Additionally, the yield of the complex was similar to that observed when we mixed the DNA and the protein before adding the oxidant.

It is also possible that protein and DNA must achieve specific orientations before they can interact and cross-link. Two observations support this proposal. First, BSA, a protein that does not bind DNA, was unable to form a covalent complex with dT_{40}. Second, when SSB or BSA alone was incubated with HOCl, only SSB formed a covalent protein−protein complex, as assessed by SDS−PAGE. Under these experimental conditions, SSB should exist in solution predominantly as a homotetramer, whereas BSA should exist as a monomer; these results strongly suggest that protein−protein cross-linking by HOCl requires intimate protein−protein interaction (*61*). Collectively, these observations suggest that both long-lived intermediates derived from HOCl and specific DNA−protein interactions are involved in the cross-linking reaction between dT_{40} and SSB.

The nature of the long-lived intermediates that promote the cross-linking reaction is uncertain. We have shown that the addition of chloramines to our DNA−protein model system can induce cross-linking. Protein-bound chloramines may deaminate into carbonyls that subsequently react with nucleophiles to form covalent cross-links (*74*). Our study shows, however, that oligomers of thymidine, adenosine, and cytidine all can participate in the cross-linking reaction. The thymidine oligomer lacks a primary amine, and the exocyclic amino groups of cytosine and adenine are poor nucleophiles (*75*). Therefore, the formation of exocyclic nucleobase chloramines is unlikely to be an intermediate step in the formation of the complex.

Chloramides are another highly reactive species (*76*). They result from the reaction of HOCl with peptides, and their production in the peptide backbone may be an initial step in the fragmentation of proteins by HOCl:

$$R-CO-NH-R' + HOCl - R-CO-NHCl-R' \quad (3)$$

It is possible that the ring nitrogens of the nucleobases react with HOCl to form chloramides that subsequently give rise

to carbon-centered radicals (*76*). Protein-bound lysine chloramines also produce both nitrogen-centered and carbon-centered radicals (*69*), raising the possibility that radical intermediates participate in DNA−protein cross-linking. Chloramides might fragment and form a carbonyl. In turn, the carbonyl might form cross-links with amines or other nucleophiles, as has been proposed for protein-bound carbonyls. It is also possible that the sugar residues of the oligonucleotides react with HOCl to generate carbonyls or other cross-linking groups (*76*).

The extent of DNA−protein cross-linking by HOCl depends on a number of variables. Approximately 0.1−0.5% of the labeled DNA was incorporated into a covalent DNA−protein complex under our standard experimental conditions. Cross-linking increased, however, with increasing HOCl concentration and incubation time. Cross-linking also increased linearly with increasing concentrations of protein or DNA (data not shown). These observations suggest that cross-linking reactions could take place in the nucleus, where the concentrations of DNA and DNA-binding proteins are much higher than those used in our experiments. Consistent with this suggestion, we observed the formation of DNA−protein cross-links in *E. coli* exposed to HOCl.

Our studies focused on the cross-linking of single-stranded DNA with SSB. It is noteworthy that double-stranded DNA in cells is generally supercoiled and coated with proteins. Double-stranded DNA may thus be relatively resistant to attack by oxidants. In contrast, single-stranded DNA is transcriptionally active and present in a much more open form, suggesting that these regions of DNA may be especially susceptible to the cross-linking reaction. Our detection of DNA−protein cross-links in bacteria, however, suggests that double-stranded DNA is also a target for damage by HOCl.

It is important to point out that the amount of cross-linking necessary to impose biological damage in a cell will depend on the repair systems available for overcoming this type of damage. The mechanisms for repairing DNA−protein cross-links in prokaryotic and eukaryotic cells are not fully understood (*37*−*39*). Most repair enzymes recognize strand breaks, altered nucleobases, or bulky lesions that disrupt the integrity of the DNA helix. Because the DNA damage in a DNA−protein cross-link is concealed by the DNA binding protein, the cell may not recognize this type of damage and may leave it unrepaired. Cis-platinum compounds induce DNA−protein cross-links; the lesions are large enough, however, to be recognized and repaired by the nucleotide excision repair pathway (*40*, *77*, *78*). In contrast, $CaCrO_4$ generates cross-links that are not repaired by the cell (*79*, *80*). It is not known whether a cell would be able to recognize and repair a DNA−protein cross-link induced by HOCl. If this lesion is left unrepaired, however, it is possible to envision that a single cross-linking event may be enough to cause cytotoxicity or mutagenicity by blocking DNA replication.

Indirect evidence suggests that damage by HOCl may not be repairable under certain conditions. Inhibition of DNA synthesis correlates strongly with cytotoxicity in bacteria exposed to HOCl (*81*−*83*). This suggests that DNA−protein cross-linking could contribute to the toxic effects of HOCl. It also underscores the possibility that the overall efficiency of DNA−protein cross-linking may not need to be great in order to affect cellular function.

DNA−protein cross-linking might exert biological effects in addition to those that could hinder DNA repair and replication. For example, cross-links might permanently fuse transcription factors to promoters or polymerases to genes, sabotaging gene expression. It also is possible that HOCl could cross-link RNA and proteins, impairing ribosome assembly and function.

Our experiments suggest that a variety of pathways involving HOCl might cross-link protein to nucleic acids in cells. HOCl is a small, membrane-diffusible molecule that presumably could reach the cell nucleus, where it could exert its effects directly at sites of DNA−protein contact. Alternatively, HOCl might first react with low molecular weight primary amines to form relatively long-lived chloramines that are much less reactive than HOCl itself. These intermediates could then diffuse into the nucleus to cross-link protein to DNA. It is also possible that HOCl could generate chloramines, and other reactive moieties, on DNA-binding proteins. Cross-linking might then occur when the modified protein subsequently interacts with DNA. Chloramines represent an attractive mechanism for protein−DNA cross-linking because they are relatively unreactive and potentially could diffuse long distances inside cells (*62*, *71*).

In summary, we have shown that HOCl, generated by myeloperoxidase, promotes the cross-linking of SSB to single-stranded homopolymers of polynucleotides. We also demonstrate that HOCl will generate cross-links between proteins and double-stranded DNA in *E. coli*. In future studies, it will be important to elucidate the chemical nature of the cross-link(s) because their detection in vivo at sites of inflammation would suggest new pathways for the mutagenic and cytotoxic effects of phagocytic oxidants.

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REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915−7922.
2. Cerutti, P. A. (1985) *Science 227*, 375−381.
3. Weitzman, S. A., and Gordon, L. I. (1990) *Blood 76*, 655−663.
4. Ananthaswamy, H. N., and Eisenstark, A. (1977) *J. Bacteriol. 130*, 187−191.
5. Imlay, J. A., and Linn, S. (1988) *Science 240*, 1302−1309.
6. McDonald, R. J., Pan, L. C., St. George, J. A., Hyde, D. M., and Ducore, J. M. (1993) *Inflammation 17*, 715−722.
7. Van Rensburg, C. E., Van Staden, A. M., Anderson, R., and Van Rensburg, E. J. (1992) *Mutat. Res. 265*, 255−261.
8. Akasaka, S., and Yamamoto, K. (1994) *Mol. Gen. Genet. 243*, 500−505.
9. Dukan, S., and Touati, D. (1996) *J. Bacteriol. 178*, 6145−61450.
10. Imlay, J. A., and Linn, S. (1987) *J. Bacteriol. 169*, 2967−276.
11. Thomas, E. L., Jefferson, M. M., Bennett, J. J., and Learn, D. B. (1987) *Mutat. Res. 188*, 35−43.
12. Bradley, M. O., Hsu, I. C., and Harris, C. C. (1979) *Nature 282*, 318−320.
13. Phillips, B. J., James, T. E., and Anderson, D. (1984) *Mutat. Res. 126*, 265−271.

14. Weitberg, A. B., Weitzman, S. A., Clark, E. P., and Stossel, T. P. (1985) *J. Clin. Invest. 75*, 1835−1841.
15. Weitzman, S. A., Weitberg, A. B., Clark, E. P., and Stossel, T. P. (1985) *Science 227*, 1231−1233.
16. Abu-Shakra, A., and Zeiger, E. (1997) *Mutat. Res. 390*, 45−50.
17. Piette, J. (1991) *J. Photochem. Photobiol. B 11*, 241−260.
18. Byun, J., Henderson, J. P., Mueller, D. M., and Heinecke, J. W. (1999) *Biochemistry 38*, 2590−2600.
19. Tamir, S., deRojas-Walker, T., Wishnok, J. S., and Tannenbaum, S. R. (1996) *Methods Enzymol. 269*, 230−243.
20. Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995) *Carcinogenesis 16*, 2045−2050.
21. Henderson, J. P., Byun, J., and Heinecke, J. W. (1999) *J. Biol. Chem. 274*, 33440−33448.
22. Henderson J. P., Byun J., Mueller D. M., and Heinecke J. W. (2001) *Biochemistry 40*, 2052−2059.
23. Henderson J. P., Byun J., Williams M. V., Mueller D. M., McCormick M. L., and Heinecke, J. W. (2001) *J. Biol. Chem.* In press.
24. Henderson, J. P., Byun, J., Williams, M. V., Mueller, D. M., McCormick, M. L., Parks, W. C., Ridnour, L. A., and Heinecke, J. W. (2001) *Proc. Natl. Acad. Sci. 98*, 1631−1636.
25. Chaudhary, A. K., Nokubo, M., Reddy, G. R., Yeola, S. N., Morrow, J. D., Blair, I. A., and Marnett, L. J. (1994) *Science 265*, 1580−1582.
26. Dizdaroglu, M. (1992) *Mutat. Res. 275*, 331−342.
27. Ames, B. N. (1989) *Free Radical Res. Commun. 7*, 121−128.
28. Angelov, D., Stefanovsky, V., Dimitrov, S. I., Russanova, V. R., Keskinova, E., and Pashev, I. G. (1988) *Nucleic Acids Res. 16*, 4525−4538.
29. Gebicki, S., and Gebicki, J. M. (1999) *Biochem. J. 338*, 629−636.
30. Meffert, R., and Dose, K. (1988) *FEBS Lett. 239*, 190−194.
31. Murthi, K. K., Friedman, L. R., Oleinick, N. L., and Salomon, R. G. (1993) *Biochemistry 32*, 4090−4097.
32. Olinski, R., Nackerdien, Z., and Dizdaroglu, M. (1992) *Arch. Biochem. Biophys. 297*, 139−143.
33. Sperling, J., and Sperling, R. (1978) *Nucleic Acids Res. 5*, 2755−2773.
34. Dizdaroglu, M., and Gajewski, E. (1989) *Cancer Res. 49*, 3463−3467.
35. Dizdaroglu, M., Gajewski, E., Reddy, P., and Margolis, S. A. (1989) *Biochemistry 28*, 3625−3628.
36. Margolis, S. A., Coxon, B., Gajewski, E., and Dizdaroglu, M. (1988) *Biochemistry 27*, 6353−6359.
37. Petit, C., and Sancar, A. (1999) *Biochimie 81*, 15−25.
38. Sancar, A., and Tang, M. S. (1993) *Photochem. Photobiol. 57*, 905−921.
39. Wood, R. D. (1999) *Biochimie 81*, 39−44.
40. Zamble, D. B., Mu, D., Reardon, J. T., Sancar, A., and Lippard, S. J. (1996) *Biochemistry 35*, 10004−10013.
41. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood 92*, 3007−3017.
42. Hurst, J. K., and Barrette, W. C., Jr. (1989) *Crit. Rev. Biochem. Mol. Biol. 24*, 271−328.
43. Klebanoff, S. J., and Clark, R. A. (1978) *The Neutrophil: Function and Clinical Disorders*, North-Holland Publishing Co., New York.
44. Harrison, J. E., and Schultz, J. (1976) *J. Biol. Chem. 251*, 1371−1374.
45. Albrich, J. M., McCarthy, C. A., and Hurst, J. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 210−214.
46. Heinecke, J. W., Li, W., Mueller, D. M., Bohrer, A., and Turk, J. (1994) *Biochemistry 33*, 10127−10136.
47. Thomas, E. L., Jefferson, M. M., and Grisham, M. B. (1982) *Biochemistry 21*, 6299−6308.
48. Winterbourn, C. C., van den Berg, J. J., Roitman, E., and Kuypers, F. A. (1992) *Arch. Biochem. Biophys. 296*, 547−555.
49. Halliwell, B. (1987) *FASEB J. 1*, 358−364.
50. Heinecke, J. W. (1999) *FASEB J. 13*, 1113−1120.
51. Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E., and Stocker, R. (1996) *J. Clin. Invest. 97*, 1535−1544.
52. Hazen, S. L., and Heinecke, J. W. (1997) *J. Clin. Invest. 99*, 2075−2081.
53. Vissers, M. C., and Winterbourn, C. C. (1991) *Arch. Biochem. Biophys. 285*, 53−9.
54. Vissers, M. C., and Thomas, C. (1997) *Free Radical Biol. Med. 23*, 401−411.
55. Heinecke, J. W., Li, W., Daehnke, H. L. d., and Goldstein, J. A. (1993) *J. Biol. Chem. 268*, 4069−4077.
56. Rakita, R. M., Michel, B. R., and Rosen, H. (1990) *Biochemistry 29*, 1075−1080.
57. Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T., and Hasegawa, E. (1986) *J. Biochem. (Tokyo) 99*, 761−770.
58. Laemmli, U. K. (1970) *Nature 227*, 680−685.
59. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Vol. 3, 2nd ed., Cold Spring Harbor Laboratory, New York.
60. Berkner, K. L., and Folk, W. R. (1977) *J. Biol. Chem. 252*, 3176−3184.
61. Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem. 63*, 527−570.
62. Thomas, E. L., Grisham, M. B., and Jefferson, M. M. (1986) *Methods Enzymol. 132*, 569−585.
63. Heinecke, J. W., Baker, L., Rosen, H., and Chait, A. (1986) *J. Clin. Invest. 77*, 757−761.
64. Zhitkovich, A., and Costa, M. (1992) *Carcinogenesis 13*, 1485−1489.
65. Morris, J. C. (1966) *J. Phys. Chem. 70*, 3798−3805.
66. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
67. Bujalowski, W., and Lohman, T. M. (1989) *J. Mol. Biol. 207*, 249−268.
68. Bujalowski, W., and Lohman, T. M. (1989) *J. Mol. Biol. 207*, 269−288.
69. Hawkins, C. L., and Davies, M. J. (1998) *Biochem. J. 332*, 617−625.
70. Thomas, E. L. (1979) *Infect. Immun. 23*, 522−531.
71. Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) *J. Clin. Invest. 70*, 598−607.
72. Costa, M., Zhitkovich, A., Gargas, M., Paustenbach, D., Finley, B., Kuykendall, J., Billings, R., Carlson, T. J., Wetterhahn, K., Xu, J., Patierno, S., and Bogdanffy, M. (1996) *Mutat. Res. 369*, 13−21.
73. Churchill, M. E., and Travers, A. A. (1991) *Trends Biochem. Sci. 16*, 92−97.
74. Hazen, S. L., Gaut, J. P., Hsu, F. F., Crowley, J. R., d'Avignon, A., and Heinecke, J. W. (1997) *J. Biol. Chem. 272*, 16990−16998.
75. Saenger, W. (1983) *Principles of Nucleic Acid Structure*, Springer-Verlag Inc., New York.
76. Hawkins, C. L., and Davies, M. J. (1998) *Free Radical Biol. Med. 24*, 1396−1410.
77. Gantt, R. (1987) *Mutat. Res. 183*, 75−87.
78. Reed, E. (1998) *Cancer Treat. Rev. 24*, 331−344.
79. Sugiyama, M., Patierno, S. R., Cantoni, O., and Costa, M. (1986) *Mol. Pharmacol. 29*, 606−613.
80. Sugiyama, M., Wang, X. W., and Costa, M. (1986) *Cancer Res. 46*, 4547−4551.
81. Grisham, M. B., Jefferson, M. M., Melton, D. F., and Thomas, E. L. (1984) *J. Biol. Chem. 259*, 10404−10413.
82. McKenna, S. M., and Davies, K. J. (1988) *Biochem. J. 254*, 685−692.
83. Rosen, H., Orman, J., Rakita, R. M., Michel, B. R., and VanDevanter, D. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 10048−10052.

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