Site-specific Oxidative DNA Damage at Polyguanosines Produced by Copper Plus Hydrogen Peroxide*

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Oxidative DNA damage has been implicated in diverse biological processes including mutagenesis, carcinogenesis, aging, radiation effects, and chemotherapy. We examined the in vitro effect of low concentrations of Cu(II) or H₂O₂ alone and in combination on supercoiled plasmid DNA. As much as 10⁻² M Cu(II) or 10⁻² M H₂O₂ alone did not break the DNA. However, a mixture of 10⁻⁶ M Cu(II) plus 10⁻⁶ M H₂O₂ produced strand breaks and inactivated transforming ability. Strand breakage was proportional to incubation time, temperature, and Cu(II) and H₂O₂ concentrations. Abasic sites were not detected. Strand breakage was inhibited by metal chelators, catalase, and by high levels of free radical scavengers implying that Cu(II), Cu(I), H₂O₂, and 'OH were involved in the reaction. The extent of DNA strand breakage was not affected by superoxide dismutase indicating that superoxide was not a major contributor to the DNA damage. DNA sequence analysis demonstrated that hot piperidine-sensitive DNA lesions were produced preferentially at sites of 2 or more adjacent guanosine residues. This sequence specificity was observed with Cu(II) plus H₂O₂ but not with Cu(I) alone. Polyguanosine sequence specificity for DNA damage induction appears to be unique among simple chemical systems. This reaction may be important in mechanisms of oxidative damage in vivo.

Oxidative DNA damage from active oxygen species has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis, radiation damage, and cancer chemotherapy. Cell metabolism has been shown to generate such oxygen species as hydrogen peroxide (H₂O₂), hydroxyl radical (-OH), singlet oxygen, superoxide, and hydroperoxyl radical (2). Trace metals such as copper and iron which are present in biological systems may interact with active oxygen species, ionizing radiation, or microwaves to damage DNA (3-8). The DNA damage is thought to be produced by a Fenton-type mechanism in which transition metal ions are cycled by first being reduced by superoxide and then oxidized by H₂O₂. The -OH produced would then damage the DNA.

In order to extend our understanding of the role of oxidative copper reactions in damaging DNA and to further characterize the reaction mechanism, we examined the effect of low levels of copper and H₂O₂ alone and in combination on supercoiled plasmid DNA. This in vitro systems is an extremely sensitive indicator of DNA damage. Our results indicate that Cu(II) in the presence of hydrogen peroxide can damage DNA through a mechanism that involves -OH radicals but not superoxide. This damage inactivates transforming ability and produces lesions that include single and double strand breaks and alterations at sites of adjacent guanosines revealed by hot piperidine treatment.

MATERIALS AND METHODS

Source of DNA and Reagents—pUC8c2 (5480 bp) DNA was purified and prepared as previously described (7, 9, 10). Cupric chloride, catalase, and superoxide dismutase were purchased from Sigma. Cuprous chloride, and 30% H₂O₂ in water were obtained from Mallinkrodt or from Fisher. In some experiments, H₂O₂ was deionized by stirring for 10 min with 20% (w/v) mixed bed ionic exchange resin (RG 501-X8, Bio-Rad). Micrococcus luteus UV-endo nuclease was obtained from Applied Genetics, Inc., (Long Island, NY) and cloned T4-endonuclease was a gift from Dr. E. Henderson, Temple University, Philadelphia, PA. Restriction enzymes were purchased from Bethesda Research Laboratories. All chemicals used were of analytical quality. Bacterial transformation was performed using routine procedures with Escherichia coli strain MBM7070 (9).

Analysis of Strand Break Introduction—DNA (0.8-1.6 µg) in 0.145 M NaCl, 0.01 M sodium phosphate, pH 7, was incubated for 30 min at 24 °C with different Cu(II) and H₂O₂ concentrations in a total volume of 12 µl. The reaction was stopped by addition of 1 µl of 0.2 M EDTA. Four µl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole, 40% sucrose in 0.4 M Tris, 0.02 M EDTA, 0.2 M sodium acetate, pH 7.8 (10 X TEA buffer)) was added and samples analyzed by electrophoresis in 0.8% agarose in 1 X TEA buffer. The gel was stained with ethidium bromide, photographed, and scanned as described previously (7).

Enzymatic Sequencing—Dideoxy sequencing was performed on double-stranded DNA with avian myeloblastoma reverse transcriptase, as previously described (9). To assess DNA damage that would terminate enzymatic polymerization of DNA (stop assay) (11). µg of pZ189 DNA was treated with copper plus H₂O₂, denatured with 0.4 M NaOH, and hybridized to about 2 ng of the pBR322 EcoR1 clockwise site primer (dGATACGACGAGGCTT), New England BioLabs, Beverly, MA. After ethanol precipitation and lyophilization, DNA was dissolved in 11 µl of water and 4 µl of 0.3 M Tris-Cl, pH 8.3, 370 mM NaCl, 2.5 mM dithiothreitol buffer. Then, 2 µl of avian myeloblastoma reverse transcriptase (10 units/µl, Promega, Madison, WI) and 40 µCl of [32P]dATP (1250 Ci/mmol, DuPont-New England Nuclear), were added. 4 µl of the previous mix were incubated 26 min at 42 °C with 1 µl of dCTP, dTTP, dGTP all at 62 µM and dATP at 3 µM (Pharmacia LKB Biotechnology Inc.). Chain termination was achieved by incubation for an additional 10 min with a solution containing 0.25 mM of each (nonradioactive) dNTP. After stopping the reaction with 6 µl of 95% deionized formamide, 10 mM EDTA, 0.2% bromphenol blue, 0.2% xylene cyanole, the sample was heated in boiling water for 3 min and loaded onto a denaturing gel containing 7% polyacrylamide with 8 M urea.

Chemical Sequencing—Conditions were established so that rapid preparation of a labeled pZ189 DNA fragment was achieved in a...
single 1.5-ml tube without the need for phenol extraction. 5 µg of pZ189 DNA in 8 µl of 50 mM Tris, 10 mM MgCl2, 0.1 mM NaCl was linearized by treatment with 5 units of EcoRI for 30 min at 37 °C. The reaction was stopped by heating 15 min at 70 °C, and then NaCl concentration was diluted in half by adding an equal volume of 15 mM Tris buffer, pH 7.6. DNA was end-labeled with 32P by incubation with 6 units of the Klenow fragment of E. coli DNA polymerase I for 60 min at 12 °C in the presence of 70 µCi of [32P]dATP (3000 Ci/ mmol, Du Pont-New England Nuclear). The reaction was chased with 1 µl of unlabeled dATP 100 mM for an additional 10 min at 12 °C and then heated for 15 min at 70 °C. A 3'-end-labeled 332-base pair fragment was generated by adding 5 units of Alul and incubating for 30 min at 37 °C. The fragment was separated by electrophoresis in a nondenaturing 5% acrylamide gel, its location in the gel determined by direct autoradiography, and the corresponding piece of gel cut out. The DNA fragment was eluted in 0.5 M ammonium acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate and 0.01 mM H2O2. The remaining form I molecules or slightly more than one single strand break/DNA molecule (data not shown). This indicates that both Cu(I) and H2O2 were required for the production of single and double strand breaks in DNA. Up to 10-2 M Cu(I) or 10-2 M H2O2 alone did not result in detectable loss of form I molecules (data not shown).

The rate of production of breaks by DNA by Cu(II) plus H2O2 as a function of incubation time was measured at different temperatures (Fig. 2). The decrease in proportion of form I DNA followed single hit kinetics as a function of incubation time with 10-4 M Cu(II) plus 10-3 M H2O2. An average of one single strand break/DNA molecule (37% form I molecules remaining) was obtained after incubation periods of 11, 52, and 300 min at 37, 23, and 6.5 °C, respectively. This represents an exponential decrease in incubation period/strand break with increasing incubation temperature.

The effect of the concentration of copper plus H2O2 on the production of DNA strand breaks was studied (Fig. 3). With 30 min of incubation at 24°C, a mixture of 0.01 M Cu(II) plus 0.01 mM H2O2 resulted in about 94% form I molecules remaining. 0.1 mM Cu(II) plus 0.1 mM H2O2 resulted in 31% remaining form I molecules or slightly more than one single strand break/5400-bp molecule. This concentration corresponds to about 1 molecule of Cu(II) and 1 molecule of H2O2/.
DNA base pair. 10 mM Cu(II) or 10 mM H₂O₂ alone did not produce any detectable loss of form I molecules (data not shown).

**Inactivation of Transforming Ability**—Low levels of Cu(II) and H₂O₂ were used to treat pZ189 at 37°C for increasing time intervals, and the ability to transform competent bacteria to ampicillin resistance was assayed (Fig. 4). Treatment with 10⁻⁶ M Cu(II) plus 10⁻⁶ M H₂O₂ for 120 min reduced transforming ability to about 37% of that with untreated plasmid. This same treatment resulted in about 60% of form I molecules remaining. This implies that the loss of transforming ability cannot be solely explained by strand breakage and suggests that additional lesions may be biologically important. Treatment with Cu(II) or H₂O₂ alone did not alter transforming ability or the proportion of plasmids in form I.

**Abasic Sites**—In an attempt to characterize further the type of damage produced by Cu(II) plus H₂O₂ treatment, abasic sites which would appear as alkali labile lesions were investigated. As a positive control, apurinic sites were produced by acid plus heat treatment. 30 min of treatment produced about one apurinic site/DNA molecule. Treated DNA was intact at neutral pH, but apurinic sites were converted to breaks when the DNA was treated with alkali (Fig. 5A). Incubation with DNase I produced strand breaks without alkali labile sites, as shown by the same curve following either neutral or alkali post-treatment (Fig. 5B). Alkali treatment of DNA treated with Cu(II) plus H₂O₂ with either (i) 66 mM NaOH (final pH 12.6) for 15 min, (ii) 100 mM NaOH (final pH 12.8) for 2 h, or (iii) 2 mM glycine/NaOH, pH 12.6 for 2.5 h (Fig. 5C) all failed to detect more single strand breaks after alkali treatment than after incubation in neutral conditions. This behavior resembles the pattern of damage obtained with DNase I (Fig. 5B), which does not produce alkali labile sites. Piperidine treatment of Cu(II) plus H₂O₂-treated DNA, under the same conditions used in sequencing experiments, resulted in extensive DNA breakage (data not shown).

Possible formation of abasic sites was further investigated by incubating Cu(II) plus H₂O₂-treated DNA with either UV-endonuclease or T4-endonuclease, both of which incise DNA at abasic sites. As a positive control both enzymes produced breaks in DNA exposed to 1000 J/m² of UV radiation. These enzymes did not reveal an increased number of breaks in Cu(II) plus H₂O₂ treated DNA (data not shown).

**Chemical Species Involved in the DNA Damage**—DNA strand breakage by 10⁻⁴ M Cu(II) plus 10⁻⁸ M H₂O₂ was completely inhibited by including native catalase in the reaction but not with heat-denatured catalase (Fig. 1). Native or denatured superoxide dismutase gave little or no protection (Fig. 1). Superoxide dismutase activity was tested for the ability to inhibit decoloration of nitro blue tetrazolium by potassium superoxide (12). As a negative control, boiling superoxide dismutase for 5 min irreversibly abolished about 90% of its native activity. As positive controls, superoxide dismutase retained about 50% of its original activity with 10⁻⁴ M Cu(II) plus 10⁻⁸ M H₂O₂ under conditions of Fig. 1 and also by treatment with 10⁻⁴ M H₂O₂ for 30 min at 23°C at pH 7. More than 95% of the activity remained after incubation with 10⁻⁴ M H₂O₂ in agreement with the values reported (13). The fact that protection against DNA strand breakage was observed with catalase but not with superoxide dismutase, implies that H₂O₂ but not superoxide was involved in the reaction. This also indicates that a superoxide-forming system was not required for production of the observed DNA scission.

Identical strand breakage was observed when H₂O₂ from two different commercial sources was used and also when the sodium stannate (up to 10 ng/ml) used as H₂O₂ stabilizer was removed by ionic exchange chromatography through a mixed bed resin (data not shown). The same results were also obtained when the reaction was carried out in saline in the absence of phosphate or Tris buffer.

The protective effect of two metal chelating agents, the divalent cation chelator, EDTA, and the specific Cu(II) chelator, bathocuproine (14), was studied. Both afforded almost complete protection (Table I) suggesting that, in addition to Cu(II), Cu(I) was also involved in production of DNA damage. We thus studied the direct effect of incubating supercoiled plasmid DNA with varying amounts of Cu(I) in the absence of H₂O₂ (Fig. 3). One single strand break/molecule was produced by incubation in 1.4 mM Cu(I) without H₂O₂ for 30 min at 24°C. At higher concentrations (5–10 mM Cu(II)), the plasmid integrity was highly disrupted (not shown). The extent of loss of form I molecules with Cu(I) was about 18 times less than with an equimolar mix of Cu(II) and H₂O₂ (Fig. 3).

The participation of ·OH radicals in DNA damage was studied by examining the protective effect of radical scavengers (Table I). Sodium azide (0.1 M) was an efficient protector. At higher concentrations several other hydroxyl radical scavengers ( tert-butyl alcohol, dimethyl sulfoxide, mannitol, ethanol, bovine serum albumin, and polyethylene glycol) were
Effect of metal chelators and hydroxyl radical scavengers on DNA strand scission induced by Cu(II) and H$_2$O$_2$

| Agent                  | Conc. | Protection* |
|------------------------|-------|-------------|
| Chelators              |       |             |
| EDTA                   | 0.1 M | 93          |
| Bathocuproine          | 0.08 M| 100         |
| Scavengers             |       |             |
| Sodium azide           | 0.1 M | 90          |
| tert-Butyl alcohol     | 0.01 M| 76          |
| Mannitol               | 0.1 M | 60          |
| Polyethylene glycol    | 1 M   | 60          |
| Ethanol                | 0.1%  | 32          |
| Dimethyl sulfoxide     | 12%   | 33          |
| Bovine serum albumin   | 10 µg/ml | 51        |
| Potassium chloride     | 1 M   | 12          |

*Protection = 1 - [(S$_{max}$ - S$_i$)/(S$_{max}$ - S$_0$)] where S$_{max}$ is % remaining supercoiled after treatment with mix plus agent, S$_i$ is % remaining supercoiled in control untreated plasmid, and S$_0$ is % remaining supercoiled with mix without agent.

DNA was incubated with avian myeloblastoma reverse transcriptase in the absence of dNTP. Some types of DNA damage, such as cyclobutane dimer photoproducts, cause termination of the polymerase reaction (15). Results obtained in seven experiments including reaction conditions like those in Fig. 6 failed to detect polymerase termination at the sites of damage detected polymerase termination at the sites of damage detected in Fig. 6 (data not shown). In control reactions UV-treated plasmids showed terminations at pyrimidine photoproducts (data not shown).

DISCUSSION

DNA Damage—Although neither Cu(II) nor H$_2$O$_2$ alone at concentration up to $10^{-2}$ M was able to damage DNA, together they formed an active DNA damaging mixture. The sensitizing effect of copper was previously reported in phage inacti-
vation after ionizing radiation (4) and in the decrease of sedimentation speed of T7 bacteriophage DNA after exposure to reduced oxygen species (16). High concentrations of H₂O₂ have been known for many years to produce single and double strand breaks (references in 17) due in part to sugar damage (3). However, using a sensitive supercoiled plasmid assay, we detected single strand breakage and loss of transforming ability induced by as little as 10⁻⁴ M Cu(II) plus 10⁻³ M H₂O₂ with 30 min of incubation at 37 °C (Fig. 4).

We found polyguanosine sequences to be preferentially damaged as revealed by scission after piperidine treatment. Feldberg et al. (17) reported that Cu(II) plus H₂O₂ was able to damage poly(dG-C) but not poly(dA-T). Guanine has been found in oxidative radiation damage to nitrogen mustards (19), antitumor agents (bleomycin, neocarzinostatin, cis-platinum) (20), and antibiotic (tetracycline) action (21). However, our finding of the preference for polyguanosine sequence damage appears to be a unique feature of the Cu(II) plus H₂O₂ system in comparison to that reported for other systems involving metals (14, 20, 22).

One type of guanosine damage might be loss of guanine as has been found in oxidative radiation damage to DNA or with 1,10-phenanthroline-copper complex (3). This would result in an abasic site. We were unable to detect abasic sites (alkalilabile sites or UV- or T4-endonuclease sensitivity) after the damage produced in DNA by Cu(II) plus H₂O₂ (Fig. 5). The fact that the polyguanosine lesion is converted to a strand break only after hot piperidine treatment (90 °C for 30 min), favors the idea of nucleotide lesion(s) stable under the usual alkali treatment, but made evident only under more drastic conditions. The guanosine damage thus might be a nucleotide modification such as the formamido pyrimidine derivative (23). Another feature of the guanosine damage is that it did not block in vitro DNA polymerization. This polymerase bypass has also been observed with the oxidative lesion, 8-hydroxydeoxyguanosine, and was found to be an error-prone process (24).

Chemical Species Involved in Inducing Damage—Both Cu(II) and H₂O₂ are required for the DNA damage since EDTA and catalase both have a protective effect (Table I). The protection by bathocuproine indicates that Cu(I) is also involved. This is further confirmed by the observation that Cu(I) alone produces single and double strand breaks in DNA in absence of H₂O₂ (Figs. 3 and 6). However, Cu(I) is about 18 times less effective in producing strand breaks than equimolar Cu(II) plus H₂O₂ (Fig. 3) and also lacks the base sequence specificity for nucleotide damage (Fig. 6). The large differences existing in the relative stabilities of the complexes and chelates of Cu(II) and Cu(I), together with the fact that Cu(II) usually forms planar compounds while Cu(I) forms tetrahedral compounds (25) may account for the different base specificity observed in our experiments.

The ability of .OH scavengers to protect DNA from damage, indicates that the .OH participates in the mechanism of strand break formation produced by Cu(II) and H₂O₂. However, only partial protection of DNA is conferred by .OH scavengers. This partial protection given by free radical scavengers suggests short action radii for the .OH, which hinder the protective efficiency of scavenger molecules. Hydroxyl radicals that possess this property by being complexed either to its precursor metal or to its substrate have been proposed in other systems (8). Superoxide radicals do not seem to play a major role in the DNA strand scission since active superoxide dimutase lacks any protective effect. This also suggests that direct reduction of Cu(II) by H₂O₂ (17) is not a principal reaction since this reaction would generate superoxide. The lack of superoxide dismutase protection in our experiments agrees with similar results on the DNA damage produced either by ascorbate-Cu(II) (4) or camphothecin-Cu(II) (14). Singlet oxygen might also play a role in this mechanism since it has been reported to react specifically with guanine and is scavenged by azide (28).

Model Mechanism—We hypothesize that DNA damage in the presence of Cu(II) and H₂O₂ occurs in a multistage mechanism that involves one step favoring base sequence specificity, the binding of Cu(II) (but not Cu(I)) to an electronegative region involving at least two guanines. Guanosine residues are the most electronegative bases, and adjacent guanosine residues would be the most electronegative regions of the DNA molecule (19). This line of reasoning is further supported by previous evidence showing binding of Cu(II) to guanine (27, 28). As a second step Cu(II) would react with DNA, perhaps through proton transfer involving guanine (29), in a similar fashion as proposed for copper and other substances (25, 27). This reduction of Cu(II) could yield oxidation products of guanine which are piperidine sensitive but are not alkali sensitive and do not block DNA polymerization in vitro. In the last step, H₂O₂ reacts with the Cu(II) formed, either still bound or in the proximity of DNA, generating .OH and regenerating Cu(II). In turn, .OH produces (additional) piperidine-sensitive base damage and/or strand breaks at short range from the original Cu(II)-binding site.

Biological Relevance—Our experiments show that DNA breaks and other lesions can be produced without the need of a superoxide-generating system or complex drugs, but with physiological concentrations (30) of simple substances such as Cu(II) and H₂O₂ that are present in a wide range of biological systems.

DNA damage involving copper would be relatively favored in diseases where copper concentration is elevated such as Menkes' syndrome (31), Wilson's disease, or certain neoplasms (32). In some neoplasms oxygen concentration is low as compared with normal cells (6). In normal cells Cu(I) would be oxidized mainly by O₂ with little .OH formation and DNA damage. In tumor cells, the oxidation of Cu(I) by H₂O₂ would be favored, resulting in the DNA damage studied in this paper. Better biochemical understanding of the mechanism involving Cu(II) and H₂O₂ might result in the development of a simpler cancer therapy with fewer detrimental side reactions.

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Cu(II) Plus H₂O₂-induced DNA Damage

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