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Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide

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Mixtures of Cu^{2+} and H_2O_2 at pH 7.4 caused damage to the bases in DNA greater than that caused by mixtures of Fe^{3+} and H_2O_2. Addition of ascorbic acid to the Cu^{2+}/H_2O_2 system caused a very large increase in base damage, much greater than that produced by the Fe^{3+}/H_2O_2/ascorbic acid system. The products of base damage in the presence of Cu^{2+} were typical products that have been shown to result from attack of hydroxyl radicals upon the DNA bases. Cytosine glycol, thymine glycol, 8-hydroxyadenine and especially 8-hydroxyguanine were the major products in both the Cu^{2+}/H_2O_2 and the Cu^{2+}/H_2O_2/ascorbic acid systems. Base damage in DNA by these systems was inhibited by the chelating agents EDTA and nitrilotriacetic acid and by catalase, but not by superoxide dismutase, nor by the hydroxyl-radical scavenger mannitol. It is proposed that Cu^{2+} ions bound to the DNA react with H_2O_2 and ascorbic acid to generate hydroxyl radicals, which then immediately attack the DNA bases in a site-specific manner. A hypoxanthine/xanthine oxidase system also caused damage to the DNA bases in the presence of Cu^{2+} ions. This was inhibited by superoxide dismutase and catalase. The high activity of Cu^{2+} ions, when compared with Fe^{3+} ions, in causing hydroxyl-radical-dependent damage to DNA and to other biomolecules, means that the availability of Cu^{2+} ions *in vivo* must be carefully controlled.

INTRODUCTION

Oxygen-derived species such as superoxide radicals ($O_2^{\cdot-}$) and H_2O_2 are produced in mammalian cells during normal aerobic metabolism (for reviews see refs. [1] and [2]). Excess generation of these species *in vivo* results in damage to many biological molecules, including DNA. Indeed, strand breakage is frequently observed in cells subjected to oxidative stress [2–4]. Oxygen-derived species are mutagenic, and may be able to act as promoters of carcinogenesis [3–12]. However, neither $O_2^{\cdot-}$ nor H_2O_2 at physiological concentrations causes any strand breakage or chemical modification of the bases in DNA [13–17]. One proposal that has been made to account for DNA damage in cells subjected to oxidative stress is that $O_2^{\cdot-}$ and H_2O_2 interact with transition-metal ions bound to the DNA, or close to it, to form highly reactive oxidizing species such as hydroxyl radicals ($^{\cdot}OH$) [3,4,18,19]. It is well-established that Fe^{3+} ions can lead to formation of $^{\cdot}OH$ from $O_2^{\cdot-}$ and H_2O_2, both *in vitro* and *in vivo* [2,4,9,17–20]. Indeed, when DNA is exposed to $O_2^{\cdot-}$ and H_2O_2 in the presence of Fe^{3+} ions *in vitro*, the pattern of base modification is very similar to that produced by ionizing radiation, an established source of $^{\cdot}OH$ [16,17].

Mixtures of Cu^{2+} ions and H_2O_2 [21,22], sometimes with added ascorbic acid [23–25] or thiols [26], have been shown to produce extensive strand breakage in DNA. Strand breakage often occurs near guanine residues, and it has been suggested that Cu^{2+} ions bind to DNA at these sites [21]. Indeed, Cu^{2+}-dependent DNA fragmentation has been reported to be much more extensive than that produced by equimolar Fe^{3+} ions in comparable reaction mixtures [23,26,27]. Several authors have suggested that Cu^{2+} ions react with H_2O_2 to produce $^{\cdot}OH$, which mediates the DNA strand breakage [22–26,28,29]. However, other researchers have disputed the formation of $^{\cdot}OH$ in reactions involving Cu^{2+} ions and H_2O_2 [30,31], and the debate continues in the literature [27,30–34].

$^{\cdot}OH$ radicals may be detected by a variety of techniques, including 'trapping' methods such as spin-trapping and aromatic hydroxylation (reviewed in refs. [2] and [35]), but the results obtained so far with systems containing Cu^{2+} ions have been inconclusive, largely owing to the complexities of the methodology [21–34]. In addition, if $^{\cdot}OH$ is formed by Cu^{2+} ions bound to DNA and then immediately attacks the DNA (the so-called 'site-specific' type of reaction [2]), it is very difficult for any trapping molecule to intercept the $^{\cdot}OH$. In the present paper, therefore, we have adopted an alternative approach, which might be called a 'fingerprinting' method [2,16,17]. When $^{\cdot}OH$ attacks DNA, it produces a wide range of products by attacking all four DNA bases (reviewed in refs. [36] and [37]). Formation of this wide range of products appears to be characteristic of $^{\cdot}OH$ attack, in that other reactive oxygen species either do not modify the DNA bases at all ($O_2^{\cdot-}$, H_2O_2, the bleomycin ferryl radical) or else they form only a few products (singlet oxygen, cytotoxic aldehydes, HOCl) ([16,17,38]; O. I. Aruoma, B. Halliwell, E. Gajewski & M. Dizdaroglu, unpublished work).

In the present work, we have used this fingerprinting method to investigate the base products formed in DNA by H_2O_2 in the presence of Cu^{2+} ions, to see whether they are typical of attack by $^{\cdot}OH$. In addition, we have examined the suggestions [23,26,27] that H_2O_2 in the presence of Cu^{2+} ions might lead to more DNA damage than in the presence of Fe^{3+} ions.

MATERIALS AND METHODS

Materials

Calf thymus DNA, ascorbic acid, mannitol, bovine copper–zinc superoxide dismutase, catalase (type C-40; thymol-free) and EDTA-free xanthine oxidase were purchased from Sigma Chemical Co. Units of superoxide dismutase were as defined by the cytochrome *c* assay [39]. One unit of catalase decomposes 1 μmol of H_2O_2/min at pH 7.0 at 25 °C, under the reaction conditions given in the Sigma catalogue. Other reagents and reference materials were as described previously [16,17,38,40].

Treatment of DNA

A stock solution of calf thymus DNA (1 mg/ml) was treated

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with Chelex resin to remove contaminating metal ions and centrifuged to remove the resin before use. The pH of the DNA solution was re-adjusted to 7.4 with Chelex-treated 1 M-HCl. Reaction mixtures contained, in a final volume of 1.2 ml, the following reagents at the final concentrations given: DNA (0.5 mg/ml), KH_2PO_4/KOH buffer (10 mM, pH 7.4) and, where indicated, $CuSO_4$ (25 μM), $FeCl_3$ (25 μM), ascorbic acid (100 μM), EDTA (100 μM), nitrilotriacetic acid (100 μM), H_2O_2 (2.8 mM), hypoxanthine (0.33 mM) or EDTA-free xanthine oxidase (0.08 unit/ml). Metal ions and chelating agents were pre-mixed, where appropriate, just before addition to the reaction mixture. Reaction mixtures were incubated for 1 h at 37 °C. Scavengers were added to the reaction mixtures, where stated, to give the final concentrations given in the Tables. After incubation, the reaction mixtures were extensively dialysed against water at 4 °C. The absorbance at 260 nm of each sample was measured in order to calculate the amount of DNA ($A_{260} = 1 \equiv 50$ μg of DNA/ml).

Hydrolysis of DNA samples, formation of derivatives of hydrolysate components and identification and quantification of derivatives by g.c.–m.s. with selected-ion monitoring were performed as described previously [16,17,38,40–42]. The column used was a fused-silica capillary column (12.5 m × 0.2 mm internal diam.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness 0.33 μm). Products derived from approx. 0.4 μg of DNA were injected on to the column for each analysis.

RESULTS

Derivatives of hydrolysed DNA samples were analysed by g.c.–m.s. with selected-ion monitoring. Products arising from free-radical attack upon the DNA bases were identified and their yields are shown in Tables 1–3. The isolated DNA used in our experiments already contained some products of base modification (Table 1), as observed previously [16,17]. Addition of H_2O_2 alone, hypoxanthine/xanthine oxidase alone, Fe^{3+} alone, Cu^{2+} alone or ascorbic acid alone produced no significant increase in the amount of base modification (results not shown). Cu^{2+}/H_2O_2 produced significant increases in the amounts of DNA base products, in contrast with the much smaller amount produced by Fe^{3+}/H_2O_2 (Table 1). The major base product formed was 8-hydroxyguanine, although increases in the amounts of almost all the other base products were observed. This wide range of base modification suggests that a highly reactive species had attacked the DNA. Addition of ascorbic acid to the Cu^{2+}/H_2O_2 system produced a striking increase in DNA damage, with 8-hydroxyguanine, 8-hydroxyadenine, cytosine glycol and thymine glycol being the major products formed. Also, very high proportional increases over the background levels were observed in the yields of 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine and 2,6-diamino-5-formamido-4-hydroxypyrimidine (about 170-fold). Similarly, the Fe^{3+}/H_2O_2/ascorbic acid system produced more DNA damage than the Fe^{3+}/H_2O_2 system. However, the amount of DNA damage by Fe^{3+}/H_2O_2/ascorbic acid was much less than that produced by the Cu^{2+}/H_2O_2/ascorbic acid system (Table 1).

Fe^{3+}-dependent DNA damage in the presence of $O_2^{\cdot-}$ and H_2O_2 is usually increased by the addition of EDTA [16,17]. It was therefore of interest to examine the effects of EDTA on Cu^{2+}-dependent DNA damage. Table 1 shows that chelation of Cu^{2+} with EDTA (at a 4:1 molar ratio of EDTA to Cu^{2+}) almost completely inhibited Cu^{2+}-dependent DNA base damage by

Table 2. Effects of superoxide dismutase and mannitol on yields of base products formed in DNA by treatment with Cu^{2+}/H_2O_2

All values represent the means ± S.D. of results from three separate reaction mixtures. Abbreviations: SOD, Cu–Zn superoxide dismutase; others as defined in Table 1 legend.

	Yield of modified base (nmol/mg of DNA)		
	DNA/Cu^{2+}/H_2O_2/Asc	DNA/Cu^{2+}/H_2O_2/Asc/SOD (10^3 units/ml)	DNA/Cu^{2+}/H_2O_2/Asc/mannitol (50 mM)
5-OH-5-MeHyd	0.43 ± 0.01	0.47 ± 0.02	0.34 ± 0.01
5-OH-Hyd	0.51 ± 0.007	0.42 ± 0.01	0.28 ± 0.02
Cyt glycol	9.05 ± 0.54	7.90 ± 0.43	8.28 ± 0.40
Thy glycol	5.06 ± 0.25	5.76 ± 0.18	5.06 ± 0.44
5,6-diOH-Cyt	1.84 ± 0.39	1.23 ± 0.01	1.50 ± 0.19
FapyAde	1.70 ± 0.15	1.45 ± 0.27	1.58 ± 0.09
8-OH-Ade	14.7 ± 0.55	14.2 ± 2.2	16.3 ± 0.11
FapyGua	1.75 ± 0.14	0.79 ± 0.12	0.89 ± 0.11
8-OH-Gua	48.2 ± 6.8	41.4 ± 14.2	53.9 ± 0.8
Total	83.2 ± 8.84	73.5 ± 17.3	88.1 ± 2.2

Table 1. Yields of base products formed in DNA by treatment with the Cu^{2+}/H_2O_2 systems

All values represent the means ± S.D. of results from three separate reaction mixtures for each column. Abbreviations: 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; Cyt glycol, cytosine glycol; Thy glycol, thymine glycol; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-5-formamido-4-hydroxypyrimidine; 8-OH-Ade, 8-hydroxyadenine; 8-OH-Gua, 8-hydroxyguanine; Asc, ascorbic acid; NTA, nitrilotriacetic acid.

	Yield of modified base (nmol/mg of DNA)								
Base product	DNA alone	DNA/Cu^{2+}/H_2O_2	DNA/Fe^{3+}/H_2O_2	DNA/Cu^{2+}/H_2O_2/Asc	DNA/Fe^{3+}/H_2O_2/Asc	DNA/Cu^{2+}–EDTA/H_2O_2	DNA/Cu^{2+}–NTA/H_2O_2	DNA/Cu^{2+}–EDTA/H_2O_2/Asc	DNA/Cu^{2+}–NTA/H_2O_2/Asc
5-OH-5-MeHyd	0.26 ± 0.001	0.55 ± 0.05	0.13 ± 0.004	0.43 ± 0.01	0.43 ± 0.02	0.29 ± 0.05	0.43 ± 0.02	0.68 ± 0.06	0.54 ± 0.05
5-OH-Hyd	0.23 ± 0.037	0.25 ± 0.022	0.09 ± 0.009	0.51 ± 0.007	0.12 ± 0.01	0.13 ± 0.01	0.18 ± 0.03	0.30 ± 0.07	0.16 ± 0.007
Cyt glycol	0.14 ± 0.03	0.56 ± 0.015	0.55 ± 0.02	9.05 ± 0.54	1.49 ± 0.06	0.29 ± 0.06	0.26 ± 0.025	1.19 ± 0.15	8.15 ± 0.81
Thy glycol	0.24 ± 0.027	0.98 ± 0.06	0.33 ± 0.03	5.06 ± 0.25	0.89 ± 0.05	0.42 ± 0.09	0.57 ± 0.027	1.22 ± 0.03	2.98 ± 0.20
5,6-diOH-Cyt	⩽ 0.01	0.08 ± 0.06	0.09 ± 0.001	1.84 ± 0.39	0.45 ± 0.04	⩽ 0.01	⩽ 0.01	0.07 ± 0.002	0.65 ± 0.11
FapyAde	⩽ 0.01	0.34 ± 0.04	0.25 ± 0.003	1.70 ± 0.15	0.76 ± 0.04	0.12 ± 0.005	0.14 ± 0.04	0.28 ± 0.03	1.81 ± 0.16
8-OH-Ade	0.40 ± 0.008	2.43 ± 0.02	0.65 ± 0.08	14.7 ± 0.55	1.49 ± 0.34	1.42 ± 0.19	1.20 ± 0.13	3.00 ± 0.28	7.94 ± 1.16
FapyGua	⩽ 0.01	0.7 ± 0.007	0.25 ± 0.006	1.75 ± 0.14	0.38 ± 0.04	⩽ 0.01	⩽ 0.01	1.00 ± 0.14	1.12 ± 0.29
8-OH-Gua	1.02 ± 0.09	9.02 ± 0.12	1.29 ± 0.09	48.2 ± 6.8	2.14 ± 0.24	2.33 ± 0.16	3.99 ± 0.71	9.40 ± 0.42	26.9 ± 4.6
Total	2.32 ± 0.19	14.3 ± 0.39	3.63 ± 0.24	83.2 ± 8.84	8.15 ± 0.84	5.02 ± 0.57	6.79 ± 0.98	17.1 ± 1.18	50.3 ± 7.39

Table 3. Yields of base products formed in DNA by treatment with the Cu^{2+}/hypoxanthine/xanthine oxidase systems

All values represent the means ± S.D. of results from three separate reaction mixtures. Abbreviations: HX/XO, hypoxanthine/xanthine oxidase system; SOD, Cu–Zn superoxide dismutase; others as defined in Table 1 legend.

	Yield of modified base (nmol/mg of DNA)				
Base product	DNA alone	DNA/Cu^{2+}/ HX/XO	DNA/Cu^{2+}–EDTA/ HX/XO	DNA/Cu^{2+}/ HX/XO/ SOD	DNA/Cu^{2+}/ HX/XO/ catalase
5-OH-5-MeHyd	0.26±0.001	0.58±0.013	0.44±0.03	0.45±0.07	0.34±0.05
5-OH-Hyd	0.23±0.037	0.38±0.004	0.32±0.03	0.21±0.08	0.30±0.07
Cyt glycol	0.14±0.03	0.75±0.10	0.41±0.04	0.36±0.08	0.23±0.04
Thy glycol	0.24±0.027	0.89±0.01	0.60±0.076	0.17±0.01	0.26±0.04
5,6-diOH-Cyt	⩽0.01	0.08±0.009	0.06±0.005	0.08±0.001	⩽0.01
FapyAde	⩽0.01	1.03±0.04	0.33±0.002	⩽0.01	0.12±0.03
8-OH-Ade	0.40±0.008	0.88±0.04	0.52±0.025	0.47±0.01	0.28±0.07
FapyGua	⩽0.01	0.82±0.01	0.62±0.06	0.16±0.02	⩽0.01
8-OH-Gua	1.02±0.09	8.57±0.95	1.38±0.14	0.51±0.04	0.47±0.05
Total	2.32±0.19	13.98±1.18	5.68±0.41	2.42±0.31	2.02±0.35

H_2O_2. Similarly, although nitrilotriacetic acid greatly stimulates Fe^{3+}-dependent DNA base damage by H_2O_2 [17], it inhibited Cu^{2+}-dependent DNA damage by H_2O_2 (Table 1). These chelating agents also markedly diminished DNA damage by the Cu^{2+}/H_2O_2/ascorbic acid system.

Table 2 shows the effect of adding scavengers of oxygen-derived species upon DNA damage by the Cu^{2+}/H_2O_2/ascorbic acid system. Superoxide dismutase sometimes showed minor and variable inhibitions of the formation of some products, but overall it had no significant effect, nor did the ·OH scavenger mannitol. Addition of catalase (10^3 units) to the reaction mixture completely inhibited the product formation, as would be expected (results not shown). This is unlikely to be a non-specific effect of protein, since the superoxide dismutase had no significant effect even though its molar concentration in the reaction mixture was greater than that of catalase.

A mixture of hypoxanthine and xanthine oxidase generates $O_2^{\cdot-}$ and H_2O_2 [39], but we found that this mixture produced no modification of the DNA bases unless Fe^{3+} ions were added to the reaction mixture [16]. Table 3 shows that Cu^{2+} ions could also promote DNA base damage by the hypoxanthine/xanthine oxidase system. Again, chelation of Cu^{2+} with EDTA had an inhibitory effect. Addition of superoxide dismutase or catalase to the reaction mixture almost completely inhibited the product formation.

DISCUSSION

A mixture of Cu^{2+} ions and H_2O_2 at pH 7.4 produced greater DNA base damage than a mixture of Fe^{3+} ions and H_2O_2. The same held true when the reducing agent ascorbic acid was added to the reaction mixture. Indeed, the H_2O_2/Cu^{2+}/ascorbic acid system produced very extensive base modification in DNA. Thus the greater ability of Cu^{2+} ions, as compared with Fe^{3+} ions, to promote DNA damage via oxygen-derived species, previously reported on the basis of studies of DNA strand breakage [23,27], was confirmed by the results on base modification in DNA presented here.

EDTA increases free-radical-induced DNA base damage by Fe^{3+} ions in the presence of H_2O_2 [17], probably largely because EDTA keeps Fe^{3+} ions in solution and favourably changes their reduction potential [43]. However, we found that EDTA is a powerful inhibitor of DNA base damage promoted by Cu^{2+} ions. The ability of EDTA to suppress reaction of Cu^{2+} ions with $O_2^{\cdot-}$ has already been reported [44]. Similarly, nitrilotriacetic acid inhibited DNA base damage in systems containing Cu^{2+} ions, although it increases the reactivity of Fe^{3+} ions [17].

The extensive DNA damage produced by the Cu^{2+}/H_2O_2/ascorbic acid system is not significantly inhibited by superoxide dismutase or by the ·OH scavenger mannitol. The observed inability of ·OH scavengers to protect against damage in various systems has often been the basis of arguments that ·OH is not responsible for that damage [2,30,31], although there are other explanations for the inability of a scavenger to protect against damage that is actually mediated by ·OH [2,33,45]. In the present case, we suggest that Cu^{2+} ions bind to the DNA and cause damage by generating ·OH in site-specific reactions [45].

The extensive pattern of DNA base modification observed (products arising from all four bases) is similar to that produced by ionizing radiation in aqueous solution [36,37,40], suggesting that Cu^{2+}-dependent DNA damage is mediated by ·OH. No other reactive oxygen species or metal ion–oxygen complex so far studied can generate this range of products from the DNA bases ([16,17,38]; O. I. Aruoma, B. Halliwell, E. Gajewski & M. Dizdaroglu, unpublished work). On the basis of the fingerprint of base damage, we therefore propose that the production of modified DNA bases by systems containing Cu^{2+} ions and H_2O_2 and/or $O_2^{\cdot-}$ or ascorbate is mediated by ·OH. This proposal does not, of course, rule out the formation of additional reactive species in systems containing Cu^{2+} ions. It has been argued that reaction of Fe^{2+} with H_2O_2 produces a ferryl species, which can then give rise to ·OH [46]. An analogous series of reactions might occur in the Cu^{2+} system, i.e., an oxo–Cu^{2+} ion complex might be a precursor of ·OH [45].

Thus, in terms of its ability to promote damage to DNA, Cu^{2+} is an extremely dangerous metal ion, much more so than Fe^{3+}. Cu^{2+} is also very efficient at promoting peroxidation of certain lipids [47,48]. These reasons may account for the fact that Cu^{2+} ions are less extensively used in the human body than Fe^{3+} ions. They may also explain why proteins able to inhibit formation of reactive radicals (i.e. ·OH) by Cu^{2+} ions in free solution are so widespread [49,50].

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recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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