1,10-Phenanthroline stimulates internucleosomal DNA fragmentation in isolated rat-liver nuclei by promoting the redox activity of endogenous copper ions

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Isolated rat-liver nuclei were incubated with a series of membrane-permeable metal-ion-complexing agents and examined for DNA damage. Of the reagents tested, only 1,10-phenanthroline (OP) and neocuproine (NC) were found to induce DNA fragmentation. Agarose-gel electrophoresis of the DNA fragments generated in the presence of OP revealed internucleosomal cleavage, which is widely considered to be a hallmark for the enzymic DNA digestion that occurs during apoptosis. Ascorbate, particularly in the presence of hydrogen peroxide, increased the levels of fragmentation induced by OP. As well as undergoing fragmentation, the DNA from nuclei was also found to contain 8-hydroxydeoxyguanosine, which indicates attack (oxidation) by the hydroxyl radical. Complementary experiments *in vitro* involving ESR determinations of hydroxyl radical formation and measurements of DNA oxidation under biomimetic conditions demonstrated that Cu^{2+}, but not Fe^{3+}, forms a complex with either OP or NC (but not the other complexing agents tested) that stimulates hydroxyl radical formation and DNA damage in the presence of hydrogen peroxide and ascorbate. It is therefore proposed that OP in the nuclei incubations binds to Cu^{2+}, which exists naturally in chromosomes, forming a complex that promotes hydroxyl-radical-dependent DNA fragmentation. These findings demonstrate the promotion of hydroxyl-radical-mediated DNA damage by endogenous Cu^{2+} and, perhaps more significantly, demonstrate that the internucleosomal DNA 'laddering' that is often used as an indicator of apoptosis may also result from DNA fragmentation by non-enzymic processes.

INTRODUCTION

Apoptosis is now widely recognized as being an important mechanism of controlled cell deletion in response to both physiological and toxic stimuli [1,2]. Internucleosomal DNA fragmentation, or DNA 'laddering', is generally recognized as being one of the most reliable biochemical markers of apoptosis and yet, with the exception of rat thymocytes, the endonucleases responsible for DNA degradation remain poorly characterized. Some endonucleases appear to be dependent on Ca^{2+} and Mg^{2+} for activity and are inhibited by Zn^{2+} (for example, the thymocyte DNase γ), whereas others are activated by a decrease in the intracellular pH [3–6].

Many of the chemical agents that induce cell killing by apoptosis also stimulate the intracellular generation of reactive-oxygen species (ROS). Menadione (2-methyl-1,4-napthoquinone) and 2,3-dimethoxy-1,4-napthoquinone, for example, generate superoxide radicals and stimulate endogenous calcium-dependent endonuclease activity [7–9]. Superoxide radicals are converted into hydroxyl radicals in the presence of redox-active metal ions, including Fe^{3+} and Cu^{2+} [10]. As hydroxyl radicals are highly reactive and will induce oxidative damage to biomolecules, including DNA, particular attention has been focused in recent years on the relative importance of direct free-radical-mediated reactions and calcium-dependent processes in cell killing during oxidative stress [11–14]. Recent findings indicate that both mechanisms of cell killing may operate, depending on the level of oxidant present. Apoptosis can occur in response to moderate oxidant concentrations, whereas necrosis, involving extensive biomolecular oxidation, is believed to occur at higher oxidant concentrations [8,9]. Several lines of evidence indicate that ROS may indeed play a physiological role in the stimulation of apoptosis [15–18]. For example, expression of the *bcl-2* proto-oncogene, which blocks apoptosis, decreases cellular ROS production [19], and antioxidants have been reported to block apoptosis in endothelial cells [20]. Furthermore it has been suggested that ROS may be responsible for the induction of immune cell deletion by apoptosis during human immunodeficiency viral infection [21]. Clearly there is a need to address the possible mechanistic links between ROS production and apoptosis.

As well as playing a central role in ROS formation, metal ions also seem to be involved in apoptosis. Indeed, a regulatory role for both redox-active (Fe^{3+} and Cu^{2+}) and non-redox-active (Zn^{2+}) metal ions in endonuclease activity and apoptosis has been suggested by several workers [4–6,22–26]. The metal-complexing agents 1,10-phenanthroline (OP) and neocuproine (NC), for example, are reported to inhibit dexamethasone- and etoposide-induced apoptosis in rat thymocytes by a mechanism involving Cu^{2+} [26], and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl-methyl)-ethylenediamine (TPEN) has been shown to stimulate apoptosis in thymocytes and lymphocytes via its chelation of Zn^{2+} [23,24]. Zn^{2+} also suppresses, in mouse thymus tissue, apoptosis induced by lipopolysaccharide [22]. Furthermore, iron deprivation induces apoptosis in HL-60 cells [25], whereas the iron-complexing agents CP-94 and desferrioxamine have no effect on dexa-

Abbreviations used: BC, bathocuproinedisulphonic acid; DABA, diaminobenzoic acid dihydrochloride; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; 8-OHdG, 8-hydroxydeoxyguanosine; NC, neocuproine; OP, 1,10-phenanthroline (*o*-phenanthroline); ROS, reactive-oxygen species; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl-methyl)ethylenediamine.

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methasone- and etoposide-induced apoptosis in thymocytes [26]. As metal-ion-complexing agents may also affect ROS formation via the activation or removal of redox-active metal ions, the present investigation was undertaken to investigate the effects of a series of these reagents on DNA fragmentation in isolated rat-liver nuclei. This system was chosen for study because it contains a Ca^{2+}/Mg^{2+}-dependent endonuclease and therefore provides a convenient model for investigating both endonuclease activation (apoptosis) and free-radical-mediated DNA damage [3].

MATERIALS AND METHODS

Chemicals

Unless stated otherwise (in cited papers), all chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.). RNase A, RNase T1 and proteinase K were from Boehringer Mannheim U.K. The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purified by vacuum distillation (Kugelrohr) and stored under nitrogen at −70 °C.

Preparation and incubation of nuclei

Rat-liver nuclei were prepared as described previously [3] from male rats (170–190 g) of either the Sprague–Dawley or Lister (Rowett Strain) breed. Both breeds gave similar results, those presented being from the latter. After the final centrifugation through the 2.3 M sucrose cushion (in 50 mM Tris/HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5)[3], nuclei from two livers were carefully suspended in 10 ml of incubation medium (0.3 M sucrose, 25 mM KCl, 25 mM Hepes, 4 mM $MgCl_2$, 2 mM K_2HPO_4, 5 mM EGTA, pH 7.0). After further centrifugation for 3 min at 700 ***g*** in a bench-top centrifuge using a swing-out rotor, the nuclei were again carefully resuspended in 2 ml of incubation medium. Nuclei were then counted using a haemocytometer and then incubated for 1 h in open microcentrifuge tubes floating in a 36 °C water bath with the additions indicated in the Figure legends. The assay of marker enzymes (glucose 6-phosphatase, 5′-nucleotidase, succinate–cytochrome *c* reductase) showed that there was less than 5% contamination by microsomes, plasma membranes or mitochondria.

Quantification of DNA double-strand breaks in nuclei

DNA double-strand breaks were measured by a method that is a modification of that described previously [3]. After incubation (5×10^6 in each 1 ml reaction), nuclei were pelleted from suspension by microcentrifugation (5 min at 1118 ***g***), resuspended in 0.5 ml of ice-cold lysis buffer (5 mM Tris/HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and left at 4 °C for approx. 30 min with occasional vortexing. The released chromatin was then pelleted by microcentrifugation at 7560 ***g*** for 15 min and any non-sedimentable DNA fragments were separated by decantation of the supernatant into another microcentrifuge tube. A further 0.5 ml of lysis buffer was then added to the chromatin pellet; 0.5 ml of 0.4 M perchloric acid was then added to both supernatant (chromatin fragments) and pellet (intact chromatin) tubes, which were then left overnight at 4 °C. Samples were then microcentrifuged for 10 min at 4480 ***g*** at 4 °C and the supernatants discarded, after which 0.5 ml of ice-cold ethanol containing 0.1 M CH_3CO_2K was added to each tube. After 20 min at 4 °C, samples were again centrifuged for 10 min at 4480 ***g***, the supernatants discarded, and 0.5 ml of ethanol added to the pellets. Samples were then heated at 60 °C for 15 min, allowed to cool to room temperature, and then microcentrifuged for 10 min at 4480 ***g***. The supernatants were discarded and the pellets washed with 0.5 ml of ethanol (15 min at room temperature followed by centrifugation for 10 min at 4480 ***g***). Pellets were then left to dry before adding an appropriate volume of freshly prepared 1.8 M diaminobenzoic acid dihydrochloride (DABA), depending upon the approximate amount of DNA expected to be present (1 ml for chromatin pellet samples and 0.1–0.2 ml for fragment samples). DABA was also added to tubes containing DNA standards, prepared by drying down aliquots of standard solutions of commercially available DNA (from salmon testes). Samples were heated at 60 °C for 30 min. A 90 ml aliquot of each sample was then added to 2 ml of 0.6 M perchloric acid and the fluorescence reading recorded using excitation and emission wavelengths of 420 nm and 520 nm respectively. The zero and 100% values on the instrument were set with appropriate DNA standards. DNA fragmentation was then expressed as the percentage of the total DNA that resisted centrifugation in lysis buffer.

Agarose gel electrophoresis

Nuclei (45×10^6 in each 1 ml reaction) were lysed and centrifuged to separate fragments as described above. The fragment samples, in 0.5 ml of lysis buffer, were then incubated for 30 min at 37 °C with 100 mg of RNase A and 50 units of RNase T1, followed by the addition of 100 mg of proteinase K and incubation for 30 min at 45 °C. DNA was then extracted from fragment samples, resolved on 1.8% (w/v) agarose gels and revealed with ethidium bromide as described previously [3]. A λ-DNA *Bst*EII digest provided molecular mass standards.

Measurement of 8-hydroxydeoxyguanosine (8-OHdG) in nuclei

Nuclei were incubated under the conditions described in the appropriate Figure legend. After incubation the nuclei were pelleted with a microcentrifuge (1118 ***g*** for 5 min) and suspended in 0.5 ml of ice-cold lysis buffer (defined above). After treatment with RNase and proteinase K, the total DNA was extracted by the method used for the preparation of DNA for electrophoresis [3]. The DNA was then washed three times with 70% (v/v) ethanol (at room temperature and with microcentrifugation at 4480 ***g*** for 10 min), after which 0.5 ml of 40 mM Tris/HCl, pH 8.5, was added to the DNA, followed by incubation for 20 min at 55 °C, to remove traces of ethanol, and then for 3 h at 37 °C and finally overnight at room temperature to dissolve the DNA. The concentration of DNA was then estimated from the absorbance at 260 nm ($A_{260} = 1$ corresponds to approx. 50 μg/ml of double-stranded DNA). Approx. 30 μg of DNA from each sample was then hydrolysed enzymically by the method of Richter et al. [27] and analysed for 8-OHdG and dG content on a Gilson HPLC system with a Supelcosil LC 18S reverse-phase analytical column and a Supelcosil LC 18S guard cartridge (Supelco, Bellefonte, PA, U.S.A.). A Coulchem model 5100A electrochemical detector with a 5021 conditioning cell and a 5010 analytical cell (ESA Analytical Ltd, Huntingdon, U.K.) was employed for the detection of 8-OHdG. The conditioning cell was set at 100 mV and the analytical cell at 400/800 mV. A Gilson holochrome UV detector was used for the detection of dG and was set at 254 nm. The mobile phase was 50 mM potassium phosphate buffer (pH 5.5) containing 10% (v/v) methanol with a flow rate of 1 ml/min.

ESR spectroscopy

The effects of the metal-ion-complexing agents on both Fe^{3+}- and Cu^{2+}-dependent hydroxyl radical formation were determined by using a biomimetic redox-cycling system in which the metal ion

(Fe^{3+} or Cu^{2+}) is first reduced by ascorbate and then is made to react with hydrogen peroxide in a Fenton-type reaction [13]. Hydroxyl radical formation was measured by ESR spectroscopy coupled to a secondary trapping technique employing the spin trap DMPO and the hydroxyl radical scavenger ethanol [13]. With this technique, $^{\cdot}OH$ formation is indicated by the detection of the characteristic six-line ESR signal from the α-ethanol radical adduct of DMPO, DMPO/$^{\cdot}CH(OH)CH_3$ (the small doublet signal present in spectra is from the ascorbyl radical). Reactions were initiated by the final addition of ascorbate to give the reagent concentrations indicated in the Figure legends. Reaction mixtures were then transferred immediately to a quartz ESR flat-cell positioned and pretuned within the cavity of a Bruker ECS 106 ESP spectrometer by using a vacuum-delivery device [28]. Spectra were recorded with the following instrument settings: modulation frequency, 100 kHz; field sweep, 80 G; modulation amplitude, 0.8 G; sweep time, 84 s; time constant, 41 ms; power, 20 mW; receiver gain, 4×10^4. Three consecutive recordings were made for each reaction and showed no significant changes in intensity. Spectra shown are from the third scan of each reaction.

DNA oxidation measurements *in vitro*

The ethidium binding assay was used to determine the effects of the metal-ion-complexing agents on the oxidation of isolated DNA by either Fe^{3+} or Cu^{2+}, using the ascorbate/hydrogen peroxide biomimetic redox-cycling system for the generation of $^{\cdot}OH$ radicals [13,29]. Reaction mixtures were prepared as described in the Figure legends, with oxidation being initiated by the final addition of ascorbate. Reactions were terminated by the addition of 400 units of catalase and the extent of DNA damage was determined as the failure of the nucleic acid to cause a 100% enhancement in the fluorescence of ethidium bromide, as described [13,29]. To avoid any possible interference with fluorescence readings by chromophores formed from the complexing agents during incubation, DNA was removed from reaction mixtures by hydroxyapatite chromatography before the addition of ethidium bromide, as described [13].

RESULTS

Isolated rat-liver nuclei were incubated with various cell-permeable metal-ion-complexing agents and then examined for DNA fragmentation. OP and NC induced fragmentation, whereas TPEN and bathocuproinedisulphonic acid (BC) were ineffective, the latter protecting the nuclei from background levels of fragmentation (Table 1). The addition of hydrogen peroxide did not significantly affect the level of DNA fragmentation induced by OP, but the inclusion of ascorbate in incubations resulted in an approx. 1.5-fold increase in fragmentation (Table 2). The inclusion of both hydrogen peroxide and ascorbate with OP resulted in the induction of fragmentation to a level that was over 2.5 times that observed in control incubations. Hydrogen peroxide and ascorbate did not induce significant fragmentation in the absence of OP (Table 2). Examination by agarose-gel electrophoresis of the DNA fragments extracted from nuclei after these incubations revealed that the DNA had been digested to oligonucleosome-sized fragments (Figure 1). The pattern of DNA fragmentation observed is identical with that reported previously after stimulation of the Ca^{2+}/Mg^{2+}-dependent endonuclease present in rat-liver nuclei [3]. As well as DNA fragmentation, 8-OHdG formation in the DNA was measured, which indicates attack by hydroxyl radicals at dG residues. The levels of 8-OHdG detected increased in response to treatment with either hydrogen peroxide plus ascorbate or OP plus ascorbate, but did not reflect the levels of DNA fragmentation observed (Table 3).

The chelators used in this study will each bind a range of metal ions, including Cu^{2+}, Fe^{3+} and Zn^{2+}. In the presence of a reducing agent and hydrogen peroxide (or simply molecular oxygen), both Fe^{3+} and Cu^{2+} can stimulate hydroxyl radical ($^{\cdot}OH$) production [10,13]. Hydroxyl radicals are highly reactive and can cause the formation of strand breaks, oxidized bases and apurinic sites in DNA. To investigate the possibility that OP and NC stimulate DNA fragmentation by modifying (by complexation) the ability of endogenous metal ions to undergo redox reactions that lead to $^{\cdot}OH$ formation, parallel ESR measurements of $^{\cdot}OH$ formation were made on a model system. As confirmed in Figure 2(a) and Figure 3(a), both Cu^{2+} and Fe^{3+} ions stimulate hydroxyl formation in the presence of ascorbate and hydrogen peroxide (Zn^{2+}

Table 1 Induction of DNA fragmentation in rat-liver nuclei by metal-ion-complexing agents

Nuclei (5×10^6 in each 1 ml reaction) were incubated for 1 h at 36 °C in incubation medium (see the Materials and methods section) supplemented with the complexing agents indicated, which were added from stock solutions dissolved in DMSO. Control incubations with appropriate concentrations of DMSO were included. After incubation, nuclei were harvested and analysed for DNA double-strand breaks as described in the Materials and methods section. Values shown are means ± S.D. for triplicate incubations and are typical of results from three nuclei preparations.

Addition	DNA fragmentation (%)
DMSO (0.5%) alone	1.52 ± 0.16
1 mM OP (0.5% DMSO)	9.15 ± 1.17
1 mM NC (0.5% DMSO)	7.09 ± 2.40
1 mM BC (0.5% DMSO)	0.69 ± 0.13
DMSO (2%) alone	2.31 ± 1.20
1 mM TPEN (2% DMSO)	2.08 ± 0.20

Table 2 Effects of ascorbate and H_2O_2 on OP-induced DNA fragmentation in rat-liver nuclei

Nuclei (5×10^6 in each 1 ml reaction) were incubated for 1 h at 36 °C in incubation medium (see Materials and methods section) containing 1 mM OP (in DMSO), or an equivalent volume of DMSO, along with hydrogen peroxide and ascorbate, as indicated. After incubation, nuclei were harvested and analysed for DNA double-strand breaks as described in the Materials and methods section. Values shown are means ± S.D. for triplicate incubations and are typical of results from three nuclei preparations.

Addition	DNA fragmentation (%)
DMSO (0.5%) alone	2.06 ± 0.44
1 mM OP (0.5% DMSO)	5.50 ± 0.19
1 mM OP (0.5% DMSO) plus 0.2 mM H_2O_2	5.99 ± 0.53
1 mM OP (0.5% DMSO) plus 1 mM ascorbate	7.48 ± 0.21
1 mM OP (0.5% DMSO) plus 0.2 mM H_2O_2 and 1 mM ascorbate	11.24 ± 1.05
0.2 mM H_2O_2 (plus 0.5% DMSO)	2.31 ± 0.16
1 mM ascorbate (plus 0.5% DMSO)	2.36 ± 0.16
0.2 mM H_2O_2 (plus 0.5% DMSO) and 1 mM ascorbate	2.34 ± 0.21

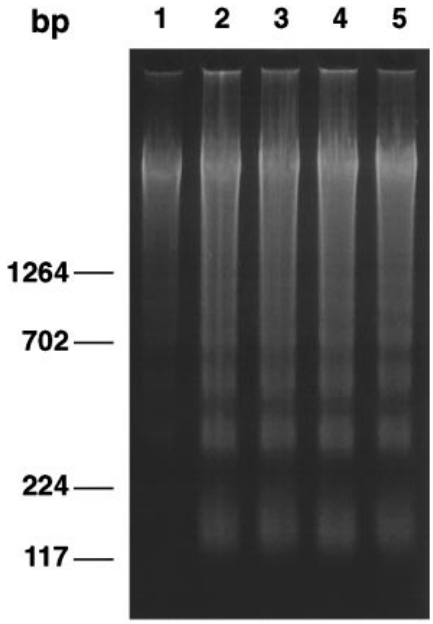


Figure 1 Resolution by agarose gel electrophoresis of DNA fragments from rat-liver nuclei treated with OP

Nuclei (45×10^6 in each 1 ml reaction) were incubated at 36 °C for 1 h in incubation medium (see the Materials and methods section) supplemented with either 0.5% DMSO (control, lane 1); 1 mM OP (with 0.5% DMSO, lane 2); 1 mM OP plus 1 mM ascorbate (with 0.5% DMSO, lane 3): 1 mM OP plus 0.2 mM hydrogen peroxide (with 0.5% DMSO, lane 4); or 1 mM OP, 1 mM ascorbate and 0.2 mM hydrogen peroxide (with 0.5% DMSO, lane 5). After incubation, DNA fragments that had been released from the chromatin during treatment in lysis buffer (see the Materials and methods section) were extracted for analysis by electrophoresis.

Table 3 Levels of 8-OHdG detected in isolated rat-liver nuclei incubated with OP, hydrogen peroxide and ascorbate

Nuclei (20×10^6 in each 1 ml reaction) were incubated for 1 h at 36 °C in incubation medium (see the Materials and methods section) containing 1 mM OP (in DMSO), or an equivalent volume of DMSO, along with hydrogen peroxide and ascorbate, as indicated. After incubation, nuclei were harvested and analysed for 8-OHdG content as described in the Materials and methods section. Values shown are means ± S.E.M. for triplicate incubations. ND, no 8-OHdG detected.

Addition	$10^6 \times$ 8-OHdG/dG
DMSO (0.5%) alone	1.10 ± 1.10
1 mM OP (0.5% DMSO)	1.77 ± 0.90
1 mM OP (0.5% DMSO) plus 0.2 mM H_2O_2	2.24 ± 1.13
1 mM OP (0.5% DMSO) plus 1 mM ascorbate	3.58 ± 1.34
1 mM OP (0.5% DMSO) plus 0.2 mM H_2O_2 and 1 mM ascorbate	0.93 ± 0.93
0.2 mM H_2O_2 (0.5% DMSO)	1.13 ± 1.13
1 mM Ascorbate (0.5% DMSO)	ND
0.2 mM H_2O_2 (0.5% DMSO) and 1 mM ascorbate	3.05 ± 0.29

ions are redox-inactive and therefore will not catalyse ·OH production). The spectrum from the Fe^{3+} reaction system (Figure 3a) is expanded on the *y*-axis 4-fold relative to the spectrum from the corresponding Cu^{2+} reaction (Figure 2a); the Fe^{3+}-catalysed reaction yielded approx. 1.5 times as much ·OH as the Cu^{2+} system. The complexing agents used were found to have distinct effects on the abilities of the two metal ions to support ·OH production. OP, for example, caused an approx. 6-fold stimulation in ·OH formation when supported by Cu^{2+} (Figure

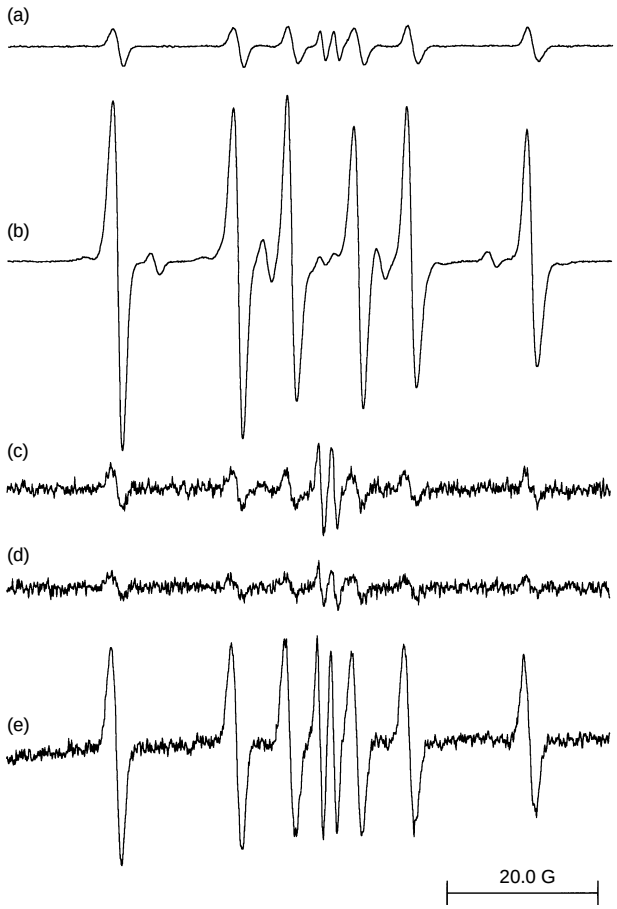
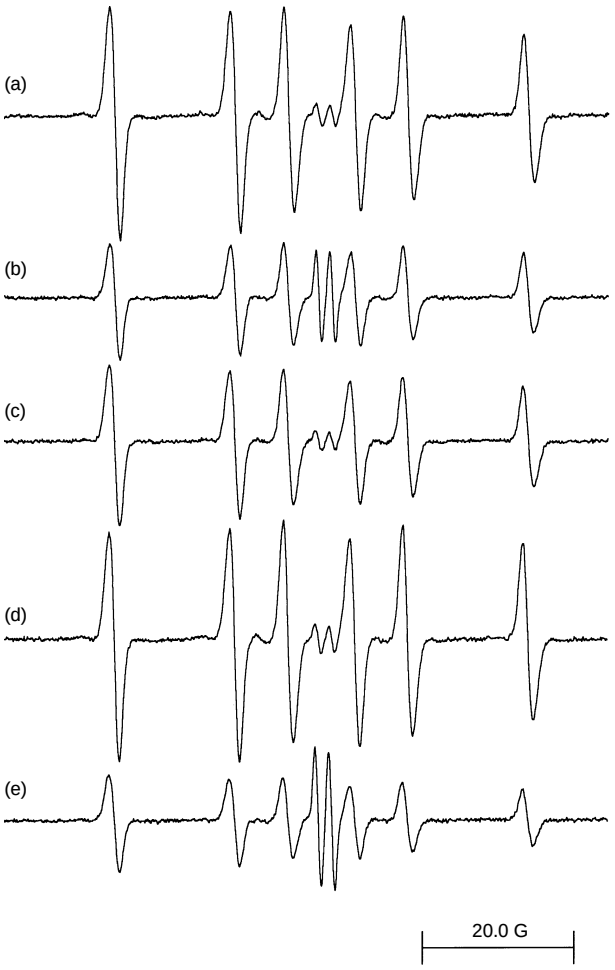


Figure 2 Effects of metal-ion-complexing agents on Cu^{2+}-dependent hydroxyl radical formation in the presence of ascorbate and hydrogen peroxide

Reactions were initiated by the addition of 1 mM ascorbate to a solution containing 1.71 M ethanol, 0.2 M Na_2HPO_4/0.1 M citric acid buffer (pH 7.0), 0.1 M DMPO, 1 mM hydrogen peroxide and 1 mM $CuCl_2$ with the complexing agents indicated: (**a**), no addition; (**b**), 5 mM OP; (**c**), 5 mM NC; (**d**), 5 mM BC; (**e**), 5 mM TPEN. With the exception of BC, which was added from a 50 mM aqueous stock solution, the complexing agents were added to reaction mixtures in the ethanol. Hydroxyl radical formation is indicated by the observation of the six-line ESR signal from the α-ethanol radical adduct of DMPO, DMPO/·CH(OH)CH_3. Spectra shown in (**c**), (**d**) and (**e**) are expanded 10-fold on the *y*-axis relative to those in (**a**) and (**b**).

2b), but suppressed formation to around one-half when supported by Fe^{3+} (Figure 3b). Indeed, with the exception of BC, which was without effect, all the complexing agents suppressed Fe^{3+}-dependent ·OH formation to a similar extent (Figure 3). In contrast with OP, TPEN, NC and BC all suppressed Cu^{2+}-dependent ·OH formation, TPEN less severely than NC and BC (Figure 2). On the basis of the observed effects of the complexing agents on Cu^{2+}- and Fe^{3+}-dependent hydroxyl radical formation alone, it is not possible to define properties unique to OP and NC and to account for their abilities to stimulate DNA fragmentation in nuclei.

It has been demonstrated previously that the level of ·OH radical formation supported by a given metal complex is not always reflected in the ability of the complex to promote oxidative damage to DNA [13]. Certain metal chelates, such as Fe–EDTA, support ·OH formation, but do not damage DNA for steric reasons. The complexes formed between Fe^{3+} or Cu^{2+} and the complexing agents employed in this study may also differ in their



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abilities to promote DNA oxidation, owing to steric factors. This was investigated *in vitro* with a well-defined model system [13,29]. Incubation of isolated DNA with 5 μM Fe^{3+} in the presence of hydrogen peroxide and ascorbate failed to induce detectable damage to the DNA, as measured by the ability of the nucleic acid to enhance the fluorescence of ethidium bromide (Table 4). The inclusion of OP in reactions resulted in the detection of slight DNA damage, whereas the other complexing agents were without effect. Although the level of damage observed in the presence of OP and 5 μM Fe^{3+} was not considered to be statistically significant, the ability of OP to support Fe^{3+}-dependent DNA damage was confirmed when the concentration of the metal ion was increased to 20 μM. No other complexing agent supported DNA damage at the higher concentration of Fe^{3+} (Table 4). Therefore the abilities of OP and NC to induce DNA fragmentation in isolated nuclei do not reflect their effects on Fe^{3+}-dependent $^{\bullet}OH$ formation (redox properties) or DNA damage

Table 4 Effects of metal-ion-complexing agents on iron-dependent oxidation of isolated DNA

Reaction mixtures contained 20 mM Mops (pH 7.0), 303 μM DNA, either 5 μM or 20 μM $FeCl_3$, 200 μM hydrogen peroxide, 200 μM ascorbate (added last) and the complexing agents indicated (each 15 μM with 5 μM $FeCl_3$ or 60 μM with 20 μM $FeCl_3$). OP, NC and BC were added from aqueous stock solutions. TPEN was dissolved in ethanol. After incubation for 30 min at 36 °C, reactions were terminated by the addition of catalase, DNA was removed from reaction mixtures on hydroxyapatite columns, and DNA oxidation was determined as the failure of the nucleic acid to cause a 100% enhancement in the fluorescence of ethidium bromide relative to undamaged DNA (see the Materials and methods section). Values given are means ± S.D. for triplicate reactions.

	Fluorescence (% DNA intact)	
Complexing agent added	5 μM Fe^{3+}	20 μM Fe^{3+}
None	101.8 ± 2.0	102.9 ± 1.7
OP	95.2 ± 2.8	88.6 ± 1.1
NC	102.7 ± 1.4	104.9 ± 6.6
BC	102.5 ± 3.6	105.7 ± 4.6
TPEN	101.5 ± 1.2	106.2 ± 0.7

(redox properties and steric factors). When similar experiments were performed with Cu^{2+} (5 μM), the findings differed in that both OP and NC enhanced DNA damage, particularly the former (Table 5). BC and TPEN were without effect (Table 5). Therefore, although OP and NC are not unique in their abilities to allow Cu^{2+}-dependent $^{\bullet}OH$ formation to proceed (formation in the presence of the latter being at relatively low levels), they are unique among the complexing agents studied in their abilities to allow Cu^{2+}-dependent DNA oxidation to proceed. Unfavourable combinations of redox properties and steric factors seem to be responsible for the failure of Cu^{2+} to induce DNA oxidation in the presence of BC and TPEN.

Although the above findings are consistent with a mechanism of DNA fragmentation involving the generation of $^{\bullet}OH$ radicals after the activation of endogenous Cu^{2+} by OP, the DNA 'laddering' that is seen when the DNA fragments are resolved on an agarose gel is more commonly associated with enzymic fragmentation, as observed during apoptosis (Figure 1). Rat hepatocytes contain a Ca^{2+}/Mg^{2+}-dependent endonuclease that is activated during apoptosis [3]. Incubation of nuclei with the nuclease inhibitor aurintricarboxylic acid (250 μM) inhibited DNA fragmentation induced by either OP or OP plus hydrogen

Table 5 Effects of metal-ion-complexing agents on copper-dependent oxidation of isolated DNA

Reaction mixtures contained 20 mM Mops (pH 7.0), 303 μM DNA, 5 μM $CuCl_2$, 200 μM hydrogen peroxide, 200 μM ascorbate (added last) and the complexing agents indicated (each 15 μM). OP, NC and BC were added from aqueous stock solutions. TPEN was dissolved in ethanol. After incubation for 30 min at 36 °C, reactions were terminated by the addition of catalase, DNA was removed from reaction mixtures on hydroxyapatite columns, and DNA oxidation was determined as the failure of the nucleic acid to cause a 100% enhancement in the fluorescence of ethidium bromide relative to undamaged DNA (see the Materials and methods section). Values given are means ± S.D. for triplicate reactions.

Complexing agent added	Fluorescence (% DNA intact)
None	106.4 ± 2.8
OP	29.9 ± 0.6
NC	66.7 ± 3.6
BC	100.2 ± 1.1
TPEN	102.7 ± 2.0

peroxide and ascorbate (results not shown), which would suggest the involvement of the endonuclease. However, aurintricarboxylic acid is expected to be a potent metal ion chelator and could therefore influence hydroxyl radical formation as well as endonuclease activity. To probe the possible involvement of calcium-dependent processes, incubations were also performed in the presence of the calcium ionophore A23187. As the incubation medium used contained millimolar concentrations of EGTA, any free calcium ions in the nuclei would be expected to be sequestered by EGTA in the medium by equilibrium through the channels formed by the ionophore. The inclusion of A23187 in incubations (10 μM) with OP failed to inhibit fragmentation, suggesting that the process is not calcium-mediated (results not shown).

DISCUSSION

The redox-active metal ions Fe^{3+} and Cu^{2+} are believed to play a central role in the promotion of hydroxyl radical formation from the partially reduced oxygen species superoxide and hydrogen peroxide, which are themselves generated in all mammalian cells [13,29]. Along with Zn^{2+}, which is non-redox-active, Cu^{2+} and Fe^{3+} have also been shown to be involved in apoptosis, which is one mechanism by which ROS can cause cell death [4–6,22–26]. In the present investigation, the metal-ion-complexing agents OP and NC are shown to induce DNA fragmentation in isolated rat-liver nuclei. Examination of the DNA fragments generated by OP on an agarose gel revealed internucleosomal fragmentation, or 'laddering', which is generally regarded as a hallmark of apoptosis.

The metal-ion-complexing agents used in this study each chelate a range of metal ions, the most relevant being Cu^{2+}, Fe^{3+} and Zn^{2+}. A comparison of the stability constants for the chelates formed between the complexing agents and these ions may give some indication as to the complexation of which metal ion leads to DNA fragmentation in nuclei. OP, for example, has cumulative affinity constants (β_3 in 0.1 M salt) for copper, zinc and iron ions of the order $Cu^{2+} \approx Fe^{2+} > Zn^{2+} > Fe^{3+}$ [30]. Caution must be exercised in the use of such values as they are for defined conditions and do not take into consideration the formation of ternary complexes between chelates and biomolecules, such as DNA. Copper ions, however, do occur naturally in chromosomes [31–35] and our finding that the addition of OP to nuclei along with ascorbate and hydrogen peroxide results in DNA fragmentation is consistent with a mechanism involving copper, rather than iron or zinc, ions: in the presence of a reducing agent (e.g. ascorbate), Cu^{2+} and OP form the $Cu^{+}(OP)_2$ complex, which binds to the DNA minor groove. On reaction with hydrogen peroxide, hydroxyl radicals are formed, resulting in DNA oxidation and strand cleavage [36,37]. We suggest that the enhancement in DNA fragmentation observed after the inclusion of ascorbate and hydrogen peroxide in incubations of nuclei with OP reflects the chemical nuclease activity of a Cu^{2+}–OP complex formed by the complexation of the endogenous metal ion. Although the possibility of a Cu^{2+}–OP complex formed between OP and contaminating exogenous Cu^{2+} cannot be ruled out, we consider this to be unlikely because the incubation medium for the nuclei contained excess EGTA, which is membrane impermeable and has a stability constant for Cu^{2+} ($\log K_1 = 17.71$) that compares favourably with that of OP ($\log K_1 = 9.30$) [30,38]. Moreover, any Cu^{2+}–OP complex formed with the exogenous metal ion would still have to bind to DNA to bring about the observed fragmentation and would therefore reflect the reactions of any endogenous DNA-bound Cu^{2+}.

As demonstrated by the ESR findings, OP markedly enhances the efficiency of Cu^{2+}-dependent $^{\bullet}OH$ formation in the presence of ascorbate and hydrogen peroxide. The possibility of iron chelation by OP can be ruled out because OP was found to suppress iron-dependent hydroxyl radical formation, believed to be due to stabilization in the ferrous state [E^0 for $Fe^{3+}/Fe^{2+} = 1.1$ V] [39]. In contrast, the complex formed between copper and OP has a redox potential [E^0 for $Cu^{2+}/Cu^{+} = 0.17$ V] that favours redox cycling, and hence $^{\bullet}OH$ generation, by ascorbate and hydrogen peroxide [39]. The finding that OP-induced DNA fragmentation is enhanced in the presence of ascorbate and hydrogen peroxide also rules out the possibility of Zn^{2+} complexation, as this metal ion will not redox cycle. The finding that OP also induces some DNA fragmentation in nuclei in the absence of added ascorbate may reflect the presence of endogenous Cu^{+} ions (Cu^{+} is stabilized in the presence of DNA) and therefore partly overcomes the need for a reducing agent in the generation of $^{\bullet}OH$ [33–35]. The detection of 8-OHdG in nuclei indicates hydroxyl radical attack on DNA. The levels of 8-OHdG detected, however, did not parallel the levels of fragmentation observed. This may be because 8-OHdG is formed by a side reaction after DNA attack by $^{\bullet}OH$. Because of its electrophilic nature, approx. 80 % of $^{\bullet}OH$ attack upon DNA is believed to occur directly at the base residues, thymine being the preferred target [40]. Strand breakage follows oxidation at the sugar moiety, which may result from direct attack by $^{\bullet}OH$ or the abstraction of a hydrogen atom on the sugar by a base radical [40]. Intramolecular redox reactions are expected to be modified by redox agents, including metal ions, hydrogen peroxide and ascorbate, and may account in part for the differential effects that these reagents were observed to have on fragmentation and 8-OHdG formation.

We believe that the evidence for the involvement of Cu^{2+}-dependent hydroxyl radical formation in our system is overwhelming: the complexing agents that induce fragmentation are the only ones to support both Cu^{2+}-dependent hydroxyl radical formation (measured by ESR) and DNA damage (as modelled *in vitro*). If DNA fragmentation resulted from the complexation of intranuclear metal ions without the need for redox cycling (ROS formation), then BC would also be effective and ascorbate and hydrogen peroxide would not enhance fragmentation. BC, in fact, suppressed background levels of DNA fragmentation in nuclei when compared with control-incubated nuclei (Table 1). Because BC prevents copper-dependent hydroxyl radical formation by stabilizing Cu^{+} [41], this further supports our conclusion that copper-dependent $^{\bullet}OH$ formation is responsible for DNA fragmentation in this system. Although we cannot exclude the possibility that hydroxyl radicals induce DNA fragmentation in nuclei indirectly, involving the activation of an endonuclease, in light of the known 'chemical nuclease' properties of $Cu^{+}(OP)_2$, this seems to entail unnecessary speculation. In support of our proposal, it has been reported elsewhere that the exposure of *Drosophila* embryo nuclei to the chemical nuclease $Cu^{+}(OP)_2$ causes DNA digestion to fragments that appear as an internucleosomal ladder on agarose gels [42].

Our results therefore demonstrate that endogenous DNA-associated Cu^{2+} may, under certain circumstances, participate in free-radical-generating reactions that result in DNA damage. Under normal circumstances, stabilization of Cu^{+} by glutathione may prevent DNA damage by the endogenous metal ion, but glutathione fails to protect DNA from Cu^{2+}-dependent damage in the presence of OP [41]. Most importantly, it appears that the internucleosomal 'ladder' of DNA fragments in agarose gels, which is widely considered to be a marker of apoptosis, may not be unique to endonuclease-mediated fragmentation and can also occur as a result of DNA fragmentation by hydroxyl radicals.

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