Histone H1- and other protein- and amino acid-hydroperoxides can give rise to free radicals which oxidize DNA

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Exposure of amino acids, peptides and proteins to radicals, in the presence of oxygen, gives high yields of hydroperoxides. These materials are readily decomposed by transition metal ions to give further radicals. We hypothesized that hydroperoxide formation on nuclear proteins, and subsequent decomposition of these hydroperoxides to radicals, might result in oxidative damage to associated DNA. We demonstrate here that exposure of histone H1 and model compounds to γ-radiation in the presence of oxygen gives hydroperoxides in a dose-dependent manner. These hydroperoxides decompose to oxygen- and carbon-centred radicals (detected by electron paramagnetic resonance spectroscopy) on exposure to Cu^+ and other transition metal ions. These hydroperoxide-derived radicals react readily with pyrimidine DNA bases and nucleosides to give adduct species (i.e. protein–DNA base cross-links). Product analysis has demonstrated that radicals from histone H1-hydroperoxides, and other protein and amino acid hydroperoxides, can also oxidize both free 2′-deoxyguanosine and intact calf thymus DNA to give the mutagenic oxidized base 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-hydroxy-2′-deoxyguanosine, 8-oxodG). The yield of 7,8-dihydro-8-oxo-2′-deoxyguanosine is proportional to the initial protein–hydroperoxide concentration, and corresponds (for histone H1-hydroperoxide, 280 μM) to approx. 1.4 % conversion for free 2′-deoxyguanosine (200 μM), and 0.14 % for 2′-deoxyguanosine in DNA (70 μg/ml). Evidence has also been obtained with DNA for reaction at cytosine and thymine, but not adenine; the lack of damage to the latter may result from damage transfer to 2′-deoxyguanosine residues. These studies demonstrate that initial radical-induced damage to nuclear proteins can give rise to subsequent DNA damage; the latter includes both DNA–protein cross-links and formation of oxidized DNA bases.

Key words: DNA damage, DNA-protein cross-links, EPR, protein hydroperoxides, protein oxidation.

INTRODUCTION

Radicals are generated in biological systems both as a by-product of normal cellular processes (e.g. from mitochondrial electron transport and some enzymatic reactions), and as a result of exposure to a number of external stimuli (e.g. high energy or UV radiation, visible light in the presence of sensitizers, thermal damage, atmospheric chemicals, transition metal ions) [1]. Most radicals react rapidly with biological targets such as lipids, nucleic acids, carbohydrates and proteins, and these reactions have been shown to affect the biological structure, function and subsequent cellular processing of these molecules [1–3]. Proteins comprise a major target within cells and thus are likely to be subject to extensive oxidation [3–5]. Reaction of proteins or amino acids with a wide range of radicals, in the presence of oxygen, has been shown to lead to the formation of both stable products [3,4] and two major types of long-lived but reactive intermediates. The latter comprise both reducing species, primarily 3,4-dihydroxyphenylalanine (DOPA), formed from oxidation of tyrosine residues [3,6,7], and oxidizing species which are mainly protein- or amino acid-hydroperoxides [6,8]. The latter are formed on a wide variety of amino acid side-chain and backbone sites [5,8–10].

In a recent study we have shown that protein-bound DOPA can give rise, in the presence of transition metal ions such as copper (which has been reported to be present in the nucleus [11,12]), to DNA damage as assessed by the formation of the oxidized DNA base 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-hydroxy-2′-deoxyguanosine; 8-oxodG [13]). This lesion is mutagenic and gives rise to G → T transversions as a result of 8-oxodG mis-pairing with adenine [14–17]; such transversions are commonly found in tumour genes [15]. Thus DOPA formation from tyrosine residues in nuclear proteins can, in the presence of transition metal ions, give rise to DNA damage.

Protein hydroperoxides (protein–OOH) and amino acid-hydroperoxides (amino acid-OOH) are long-lived in the absence of oxidizing or reducing agents, light, heat or transition metal ions [5,6,8,18], but are rapidly decomposed when treated with any of these agents. Two-electron reduction of these hydroperoxides (e.g. with borohydride) gives the corresponding unreactive alcohols. Some of these hydroxylated products have been characterized [18–21], and shown to be useful markers of oxidative damage to proteins both *in vitro* and *in vivo* [3,4]. In contrast, exposure of the hydroperoxides to light, heat or transition metal ions (e.g. Cu^{2+}, Cu^+, Fe^{2+} or Fe^{3+}) results in the formation of further reactive radicals, including alkoxyl (e.g. reaction 1), carbon-centred (from rearrangement or fragmentation of the initial alkoxyl species, reaction 2), and peroxyl (e.g. reaction 3) species [9,10].

$$\text{Protein–OOH} + Cu^+ \rightarrow \text{Protein–O}^{\bullet} + HO^- + Cu^{2+} \quad (1)$$

$$\text{Protein–O}^{\bullet} \rightarrow \text{Protein–C}^{\bullet} \quad (2)$$

$$\text{Protein–OOH} + Cu^{2+} \rightarrow \text{Protein–OO}^{\bullet} + H^+ + Cu^+ \quad (3)$$

Abbreviations used: amino acid–OOH, amino acid hydroperoxides; dG, 2′-deoxyguanosine; DMPO, 5,5-dimethyl-1-pyrroline-*N* oxide; DOPA, 3,4-dihydroxyphenylalanine; EPR, electron paramagnetic resonance spectroscopy; *G*, radiation yield (number of species formed per 100 eV absorbed energy); HPLC, high performance liquid chromatography; MNP, 2-methyl-2-nitrosopropane; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-hydroxy-2′-deoxyguanosine); protein–OOH, protein hydroperoxides.

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Previous studies have shown that protein- and amino acid-hydroperoxides can give rise to further oxidative damage. Thus protein-hydroperoxides have been shown to oxidize antioxidants and reducing agents such as ascorbate and GSH ([6,18]; P. E. Morgan, C. L. Hawkins and M. J. Davies, unpublished work), react with methionine residues [5], inactive glutathione reductase [22], oxidize lipids (M. J. Davies, S. Fu, J. A. Irwin, R. T. Dean, unpublished work) and crosslink with DNA [23]. The mechanisms of these processes are poorly characterized and it is not clear, in most cases, whether these reactions are molecular (non-radical) or radical-mediated reactions. It might be expected that radical species formed from protein- and amino acid-hydroperoxides would give rise to mutagenic lesions on DNA (such as 8-oxodG), as a wide variety of other radicals including HO$^{\bullet}$, low-molecular-weight alkoxyl (RO$^{\bullet}$, e.g. from *tert*-butyl and cumene hydroperoxides) and carbon-centred radicals (R$^{\bullet}$, e.g. hydroperoxides and hydrazines) have been shown to oxidize nucleic acids and give rise to oxidized bases, base adducts, strand breaks and protein–DNA cross links ([2,24–27]).

We hypothesized that due to the intimate interaction between histone proteins and DNA, the formation of hydroperoxides on such proteins and their subsequent decomposition to radicals, might give rise to DNA damage. We have therefore examined the formation of hydroperoxides on histone H1, as a result of exposure to γ-irradiation in the presence of O_2, the decomposition of these hydroperoxides to radicals, and explored the potential role of such radicals in inducing DNA damage (as assayed by both EPR spectroscopy and product analysis). The studies with histone H1-hydroperoxide have been complemented with studies using other protein and amino acid hydroperoxides (melittin and lysine hydroperoxides respectively) in order to examine the effect of steric and charge factors on these reactions.

MATERIALS AND METHODS

Materials

Histone H1 (subgroup f1 – lysine-rich fraction, calf thymus), DNA (calf thymus), melittin (70%), catalase (bovine liver, 15000 units/mg), MNP, and ebselen were purchased from Sigma (St Louis, MO, U.S.A.). Solutions of calf thymus DNA were incubated overnight at 4 °C in the presence of Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA, U.S.A.) to remove trace metal ions and subsequently filtered through a 5 μm filter before use. Pronase (a mixture of non-specific proteases from *Streptomyces griseus*, 7 units/mg) was supplied by Boehringer–Mannheim GmbH (Mannheim, Germany). L-Lysine HCl and DMPO were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); the latter was purified before use by stirring with activated charcoal. All buffers and aqueous solutions were made up using Nanopure water purified using a four-stage Milli-Q system (Millipore–Waters, Australia) with a 0.2 μm final filter; concentrations stated in the text are final concentrations after addition of all reagents. Microcon™-3 (3000 Da cut-off) microconcentrators were supplied by Millipore Corporation (Bedford, MA, U.S.A.). All other chemicals and HPLC solvents were ACS or AR grades and purchased from Sigma (St Louis, MO, U.S.A.), Merck (Darmstadt, Germany) or ICN (Costa Mesa, CA, U.S.A.). 8-oxodG standard was a gift from Dr. Jean Cadet (Centre d'Etudes Atomiques, Grenoble, France).

Formation of protein-hydroperoxides and amino acid-hydroperoxides via γ-irradiation

Solutions of histone H1 (4 mg/ml), melittin (2 mg/ml), and lysine (4 mM) in continuously oxygenated 10 mM sodium phosphate buffer, pH 7.4, were γ-irradiated using a ^{60}Co source at a dose rate of approx. 19 Gy·min to total doses between 250 and 2000 Gy. Immediately post-irradiation, catalase (150 units/ml) was added to eliminate H_2O_2 formed during irradiation. Protein- and amino acid-hydroperoxides have been previously shown not to be degraded by such treatment [6,8]. Solutions were subsequently aliquoted and stored in the dark at −80 °C until use. No reduction in the concentration of the histone H1-hydroperoxides was detected in preparations stored under these conditions for up to 6 months (data not shown). Hydroperoxide concentrations were determined using an iodometric assay as described previously [20].

EPR spectroscopy

Radicals were detected by electron paramagnetic resonance (EPR) spectroscopy using a Bruker EMX X-band spectrometer equipped with 100 kHz modulation and either a standard rectangular ER 4102ST or cylindrical ER 4103TM cavity. Samples (at 20 °C) were contained in standard, flattened, aqueous solutions cells. Recording of signals was commended 120 s after initiation of the reaction unless stated otherwise. Hyperfine coupling constants were measured directly from field scans and confirmed by computer simulation using the program WINSIM [28]; the latter was also used to obtain relative radical adduct concentrations. Correlation coefficients between experimental and simulated spectra were typically greater than 0.95.

Reduction of histone H1-hydroperoxides by ebselen/GSH

Reduction of histone H1-hydroperoxides to the corresponding alcohols (hydroxides) was performed by incubation with ebselen (83 μM; from a 10 mM stock solution in ethanol) and 3.3 mM GSH at 37 °C for 15 min. Excess ebselen and GSH were subsequently removed via centrifugation through Microcon-3 microconcentrators at 7500 ***g*** for 60 min. The retentate containing the histone alcohols was collected and stored at −20 °C until use.

Oxidation of dG and DNA by protein- and amino acid-hydroperoxides in the presence of Cu^+

Histone H1-, melittin- or lysine-hydroperoxide solutions (40–280 μM hydroperoxide) were added separately to solutions of 2′-deoxyguanosine (200 μM) or purified (see above) calf thymus DNA (60–80 μg/ml). Reaction was initiated by addition of Cu^+ [formed *in situ* by sequential addition of deoxygenated solutions of $CuSO_4$ (150 μM) and $TiCl_3$ (100 μM)] and incubated at room temperature for 5 min (experiments with dG) or 10 min (for DNA). Reaction mixtures containing dG were then stopped by the addition of 1 volume of chloroform, the samples vortexed briefly, and then centrifuged at 15000 ***g*** for 2 min. The upper aqueous layer was collected and purified by centrifugation through Microcon-3 microconcentrators at 9000 ***g*** for 15 min. The eluates were then concentrated to dryness using a vacuum dessicator and resuspended in 100 μl H_2O prior to HPLC analysis. Incubations containing DNA were stopped by the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of ice-cold ethanol. The DNA was then precipitated by storage at −80 °C for a minimum of 30 min, and pelleted by centrifugation at 15000 ***g*** for 30 min at 4 °C. The DNA pellets were then air-dried, resuspended in 100 μl H_2O, and hydrolysed as described previously [13].

HPLC analysis of 8-oxodG

Detection and quantification of base modifications was per-

formed using an HPLC system consisting of a SIL-10A auto injector (Shimadzu, Kyoto, Japan), two LC-10AT pumps (Shimadzu, Kyoto, Japan), a SPD-10A UV-VIS detector (Shimadzu, Kyoto, Japan), and a 5100A Coulochem II electrochemical detector with an in-line dual-electrode analytical cell (5011, ESA, Chelmsford, MA, U.S.A.). Data were digitized using a CBM-10A communications bus module interface (Shimadzu, Kyoto, Japan) and processed on a PC [13].

Samples were separated using a Zorbax ODS column (Analytical, 4.6 mm × 250 mm, 5 μm; Rockland Technologies, Inc.), maintained at 30 °C using a Waters temperature control module (Millipore–Waters, Australia), using isocratic elution (50 mM potassium phosphate, pH 5.5 and methanol; 84:16 v/v) at a flow rate of 1 ml/min for 15 min. The electrochemical detection potentials were set at +50 mV and +400 mV for the first and second electrodes respectively [13]. Under these conditions authentic 8-oxodG eluted with a retention time of 4.5 min. Peak identification was carried out on the basis of retention time, comparison with authentic 8-oxodG, and sample spiking. Calibration of the detector peak area response was carried out using a range of pure 8-oxodG standards with a sensitivity limit of approx. 1 pmol of 8-oxodG. Unmodified nucleosides and dG standards (retention time 3.8 min) were monitored using UV detection at 254 nm, and the amount of injected base or DNA calculated after calibration using the peak areas of a range of dG standard samples. Statistical analysis was carried out using *t* tests within the MYSTAT statistical applications program.

RESULTS

Detection of lysine-, melittin- and histone H1-hydroperoxides

γ-Irradiation of oxygenated solutions of histone H1 gave histone H1-hydroperoxides in a dose-dependent manner (Figure 1). At the highest radiation doses used (2000 Gy), histone H1 yielded approx. 330 ± 55 μM hydroperoxides; melittin and lysine solutions irradiated to an identical dose gave approx. 61 μM and approx. 47 μM hydroperoxides, respectively.

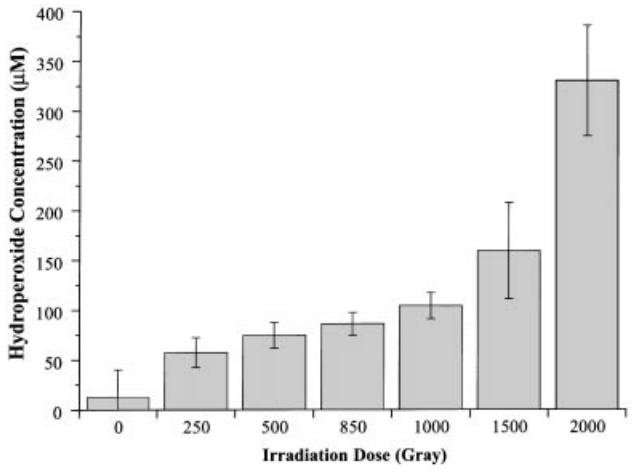


Figure 1 Yield of hydroperoxide groups formed on histone H1 after γ-irradiation in the presence of O_2 at different total radiation doses

The yield of hydroperoxide groups was determined by iodometric assay; see the Materials and methods section. Histone H1 (4 mg/ml in 10 mM sodium phosphate buffer, pH 7.4) was irradiated using a ^{60}Co source in the presence of O_2 at a dose rate of approx. 19 Gy/min to the total dose stated. Immediately post-irradiation, catalase (150 units/ml) was added to eliminate H_2O_2 formed during the irradiation process (see the Materials and method section). Values are means ± S.D. (n = 22 determinations).

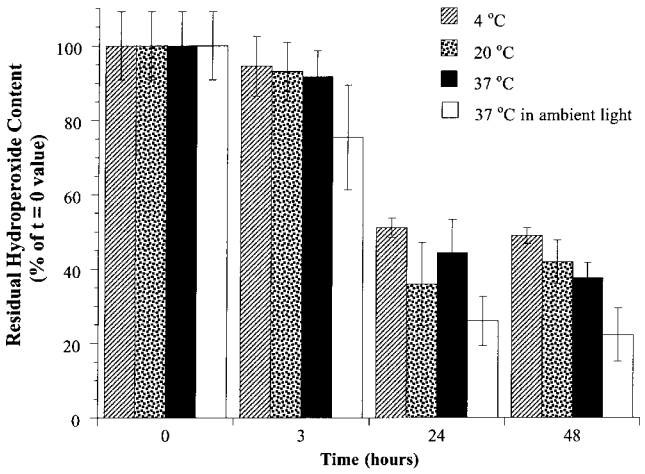


Figure 2 Decay of histone H1-hydroperoxides on incubation at different temperatures in the absence or presence of fluorescent laboratory light

The yield of hydroperoxide groups was determined by iodometric assay (see the Materials and methods section). Initial hydroperoxides were generated by ^{60}Co γ-irradiation in the presence of O_2 as reported in Figure 1 and the Materials and methods section. Samples incubated in the absence of light unless stated otherwise. Values are means ± S.D. (n = 3 determinations).

Stability of histone H1-hydroperoxides

In the absence of light, heat or transition metal ions, histone H1-hydroperoxides have a half life of approx. 24 h at 4 °C in the dark (Figure 2). The rate of decay of these hydroperoxides was accelerated at 20 or 37 °C, and increased marginally on exposure to fluorescent laboratory light (Figure 2). In all cases the decay kinetics were complex with a rapid initial loss followed by a slower secondary phase with approx. 30% of the initial hydroperoxide concentration still present after 24 h.

Formation of radicals from protein- and amino acid-hydroperoxides

A Cu^{2+}/Ti^{3+} redox couple (150 and 100 μM, respectively) was employed to generate Cu^+ *in situ* as this couple yields Cu^+ rapidly and stoichiometrically [29]. Reaction of Cu^+, formed by this method, with histone H1-, melittin- or lysine-hydroperoxides (typically 240, 50 and 38 μM respectively) in the presence of the spin trap DMPO (150 mM) at pH 7.4 and 20 °C resulted in the detection of a number of radical adduct signals by EPR spectroscopy. Figure 3 shows representative spectra. In the absence of the irradiated samples only signals from the well-characterized spin adduct DMPO-OH were detected; this species is believed to arise from autoxidation of Cu^+ or nucleophilic reactions [30]. Omission of either Cu^{2+} or Ti^{3+} resulted in the detection of weaker, but otherwise identical, signals to that observed with the complete system; the detection of these signals is ascribed to (slower) reduction of the hydroperoxides by the individual metal ions as detected with other systems [25,26]. Replacement of the irradiated samples with non-irradiated substrates, or use of irradiated samples which had been subject to reduction with

Table 1 EPR parameters of protein radical adducts detected on reaction of protein- and amino acid-hydroperoxides with Cu^+ in the presence of the spin trap DMPO

See text and the Materials and methods section for further experimental details.

Substrate	Spin adduct assignment	Hyperfine coupling constants (mT)*		Relative radical concentration (% of total)†
		a(*N*)	*a*(*H*)	
Histone H1-hydroperoxide	Carbon-centred‡	1.536	2.034	47 ± 9
	Carbon-centred§	1.564	2.266	26 ± 12
	Carbon-centred	1.569	2.353	18 ± 11
Melittin-hydroperoxide	Carbon-centred‡	1.537	2.034	56 ± 11
	Carbon-centred§	1.578	2.257	17 ± 11
	Carbon-centred	1.576	2.342	24 ± 7
Lysine-hydroperoxide	Carbon-centred	1.563	2.252	52 ± 13
	Carbon-centred	1.555	2.418	17 ± 9

* Typically ± 0.02 mT.

† Mean percentage values ($n = 10$ determinations, from computer simulations of experimental spectra, see the Materials and methods section) of total radical adduct concentration detected; values do not sum to 100% due to minor contributions from spin-trap degradation products.

‡ Assigned to adduct species with partial structure •C(O)NHR arising from bakckbone cleavage; see text for further details.

§ Possibly due to lysine side-chain-derived radicals.

ebselen/GSH (see the Methods section), did not give any substrate-derived signals. The radical adducts detected were stable for a considerable period of time (> 30 min). The signals detected with each hydroperoxide have been assigned to particular types of spin adducts on the basis of previous data [9,10]. The hyperfine coupling constants of these adducts, together with their relative concentrations (obtained from computer simulations, which were also used to confirm the experimentally-determined coupling constants), are collected in Table 1.

Histone H1 hydroperoxide (Figure 3a) gave signals which are qualitatively similar to those observed with melittin (Figure 3c), though the ratio of the species present are somewhat different (Table 1). With histone H1 (and a lesser extent melittin) both sharp and somewhat broadened signals are observed. This broadening is believed to arise from the trapping of large, protein side-chain-derived, adducts which tumble slowly in solution (cf. studies with other proteins [9,10,31,32]) with the local environment of the radical adduct providing a restriction to free motion [31]. The sharp signals detected are assigned to DMPO-OH and (one or more) species with partial structure •C(O)NHR arising from cleavage of the backbone of the protein. These species exhibit considerable freedom of motion. The mechanism of formation of the latter species has been discussed previously [10]. The absorptions assigned to protein side-chain-derived carbon-centred radicals show evidence of being due to multiple species (i.e. are composite absorptions) which have slightly different coupling constants. Some of these adducts have identical hyperfine coupling constants to those obtained from free lysine as might be expected from the high lysine content (approx. 30 mol%) of histone H1. These radicals are believed to arise via rearrangement/fragmentation reactions of an initially formed, but undetected, alkoxyl radical (cf. reactions 1 and 2; [9]). The signals detected with lysine (Figure 3e) are assigned to (at least) two carbon-centred side-chain-derived radical adducts, together with DMPO-OH [9]. No backbone(α-carbon)-derived radicals were detected with lysine (cf. previous data [3,10]).

Further information on the spin adducts detected with histone H1 and melittin hydroperoxides was obtained by digestion of the spin adducts with Pronase (17 units/ml, 37 °C, 5–15 min). This treatment releases low-molecular-mass materials from the proteins, some of which have the spin trap attached; these species tumble rapidly in solution and therefore yield sharper EPR lines (Figures 3b and 3d respectively; cf. [31–33]); this effect was less marked with melittin due to the sharper nature of the initial lines. The decreased signal intensity observed in these spectra is believed to be due to an increased rate of radical adduct decay on release from the protein. Analysis of these spectra allowed the identification of at least three carbon-centred radicals from irradiated histone H1.

The nature of the amino acid- and protein-hydroperoxide-derived radicals was explored further in experiments with the alternative spin trap, 2-methyl-2-nitrosopropane (MNP). This trap gives detectable spin adducts with a narrower range of radicals than DMPO (chiefly carbon-centred radicals), but potentially more information, as a result of the direct bonding of the radical to the nitroxide nitrogen. Unfortunately, use of this trap (50 mM or 7.5 mM) gave only weak signals from carbon-centred radicals (data not shown, cf. previous studies [9]).

Reaction of amino acid- and protein-hydroperoxide-derived radicals with DNA and its components

Reaction of the hydroperoxide-derived radicals with components of DNA was investigated by the inclusion of these materials in the above EPR spin-trapping systems. With DMPO as the spin trap, inclusion of high concentrations of each of the nucleosides resulted in characteristic changes in the nature of the carbon-centred species present (Figure 4). These changes are ascribed to reaction to hydroperoxide-derived radicals (either alkoxyl or carbon-centred) with the added nucleoside, and the subsequent trapping of the nucleoside-derived radicals. The hyperfine coupling constants of these radicals (see legend of Figure 4), identify these nucleoside-derived species as carbon-centred radicals, but do not provide information as to the exact identity of these intermediates.

Experiments with MNP allowed different nucleobase and nucleoside-derived carbon-centred radicals to be distinguished. Inclusion of uracil, thymine or cytosine, individually, into reaction systems containing the hydroperoxides, Cu^+ and MNP

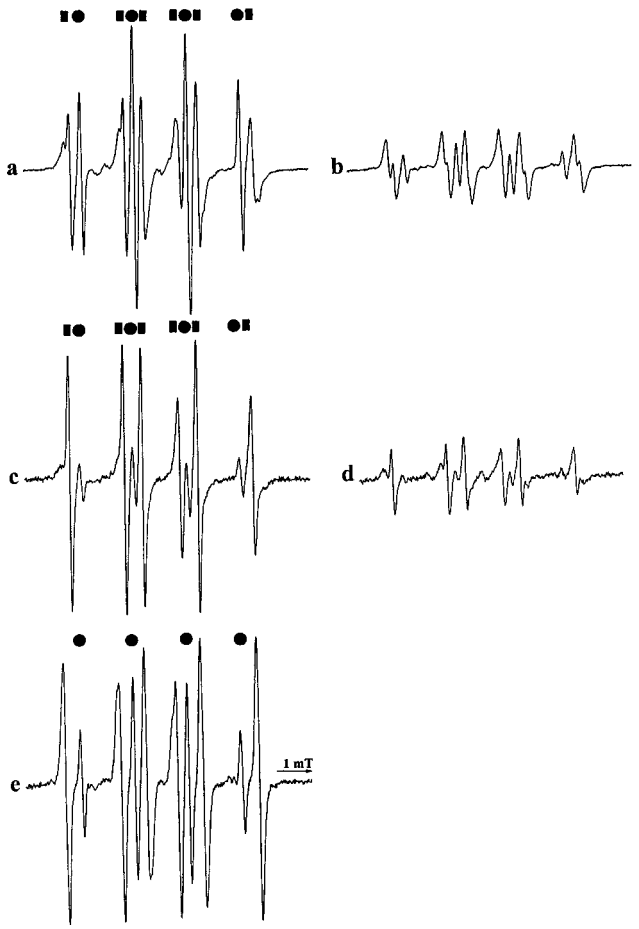


Figure 3 EPR spectra detected on reaction of histone H1-, melittin- or lysine-hydroperoxides with Cu^+ in the presence of the spin trap DMPO

Hydroperoxides were generated by ^{60}Co γ-irradiation in the presence of O_2 and assayed as reported in Figure 1 and the Materials and methods section. Reaction was initiated by addition of Cu^+ [formed *in situ* by the addition of deoxygenated solutions of Cu^{2+} (150 μM) followed by Ti^{3+} (100 μM)] to aqueous solutions of histone H1-, melittin- or lysine-hydroperoxides (typically 240, 50 and 38 μM, respectively) in the presence of the spin trap DMPO (150 mM) at pH 7.4 and 20 °C. See the Materials and methods section for further experimental details. (a) Histone H1-OOH, (b) as (a) except after pronase digestion (17 units/ml, 10 min, 37 °C) of the sample in (a). (c) Melittin-OOH. (d) as (c) except after pronase digestion (17 units/ml, 10 min, 37 °C) of the sample in (c). (e) Lysine-OOH. Typical EPR spectrometer settings: gain 1×10^6, modulation amplitude 0.025 mT, time constant 81 ms, scan time 83 s, centre field 347.5 mT, field scan 8 mT, with 8 scans accumulated. Lines indicated with (●) are assigned to DMPO-OH, lines marked (■) to •C(O)NHR radical adducts; other lines are assigned to carbon-centred radical adducts; see text and Table 1 for further details.

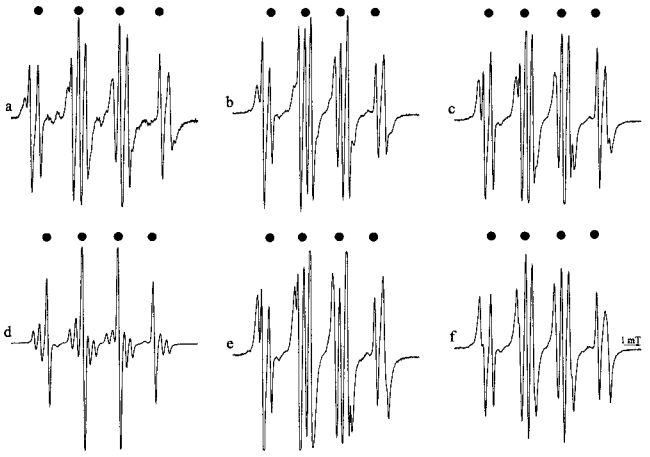


Figure 4 EPR spectra detected on reaction of histone H1-hydroperoxides with Cu^+ and the spin trap DMPO in the absence and presence of added nucleosides

Histone H1 hydroperoxides were generated by ^{60}Co γ-irradiation in the presence of O_2 and assayed as reported in Figure 1 and the Materials and methods section. Reaction was initiated by addition of Cu^+ [formed *in situ* by the addition of deoxygenated solutions of Cu^{2+} (150 μM) followed by Ti^{3+} (100 μM)] to aqueous solutions of histone H1-hydroperoxides (typically 240 μM) in the presence of the spin trap DMPO (150 mM) at pH 7.4 and 20 °C. See the Materials and methods section for further experimental details. (**a**) Histone H1-OOH with no added nucleoside. Lines indicated with (●) are assigned to DMPO-OH; for details of other assignments, hyperfine coupling constants, and relative radical adduct concentrations see Table 1. (**b**) As (a) except in the presence of adenosine; signals assigned to DMPO-OH and two carbon-centred radicals with a(N) 1.534, a(H) 2.032 mT and a(N) 1.578, a(H) 2.241 mT in relative ratio approx. 50:50. (**c**) As (a) except in the presence of guanosine; signals assigned to DMPO-OH and two carbon-centred radicals with a(N) 1.539, a(H) 2.037 mT and a(N) 1.566, a(H) 2.309 mT in relative ratio ca. 60:40. (**d**) As (a) except in the presence of cytidine; signals assigned to DMPO-OH and two carbon-centred radicals with a(N) 1.530, a(H) 2.005 mT and a(N) 1.531, a(H) 2.498 mT in relative ratio approx. 65:35. (**e**) As (a) except in the prsence of thymidine; signals assigned to DMPO-OH and two carbon-centred radicals with a(N) 1.538, a(H) 2.031 mT and a(N) 1.558, a(H) 2.269 mT in relative ratio approx. 55:45. (**f**) As (a) except in the presence of uridine; signals assigned to DMPO-OH and two carbon-centred radicals with a(N) 1.526, a(H) 2.036 mT and a(N) 1.558, a(H) 2.235 mT in relative ratio approx. 35:65. In (b)–(f) the first of the two carbon-centred radicals has parameters similar to the •C(O)NHR radical adduct detected in the absence of added nucleoside (see Table 1), and a similar assignment is therefore postulated; the second carbon-centred radical adduct detected in each case is assigned to a nucleoside-derived radical of unknown structure. Typical EPR spectrometer settings: gain 1×10^6, modulation amplitude 0.025 mT, time constant 81 ms, scan time 83 s, centre field 347.5 mT, field scan 8 mT, with 8 scans accumulated.

(concentrations as above) gave rise to different spectra to those detected in the absence of these materials. No additional signals were detected with the purines (adenine/guanine) or their nucleosides or nucleotides; this is believed to be due to either a slow rate of trapping by MNP of (delocalized) radicals generated from these substrates, or the instability of the adducts formed.

Figure 5 shows representative EPR spectra obtained in the presence of uridine (saturated solution) with each hydroperoxide, together with control spectra in the absence of uridine. Use of non-irradiated substrates gave only signals from the spin-trap degradation product di-*tert*-butyl nitroxide [$(^tBu)_2NO^•$]. The additional absorption lines detected in the presence of the nucleobases/nucleosides are assigned to nucleobase-derived species on the basis of their hyperfine coupling constants and comparison with previous data from related radicals [25,34,35]. These hyperfine coupling constants, and the relative concentrations of these species (from computer simulations), are given in Table 2. In most cases a sharp triplet signal due to $(^tBu)_2NO^•$ was also detected. The broad lines detected with histone H1- and melittin-hydroperoxides are consistent with the addition of a large, protein-derived, radical to the pyrimidine base, rather than a low-molecular-mass species. With uridine and histone H1-hydroperoxide, two major, and one minor, adducts were detected. The first of these (approx. 63% of the total spin adduct concentration), is assigned to a radical formed by addition of a hydroperoxide-derived radical to the C_6 end of the C_5–C_6 double bond of the base with subsequent trapping of this radical through the C_5 position to give the uridine C_5-yl adduct. The second species (approx. 14% of the total radical concentration) is assigned to the alternative base adduct formed by addition at the C_5 end of the C_5–C_6 double bond and subsequent trapping via the C_6 position (i.e. the C_6-yl radical). The third adduct (approx. 20% of the total radical concentration) is assigned to an acyl

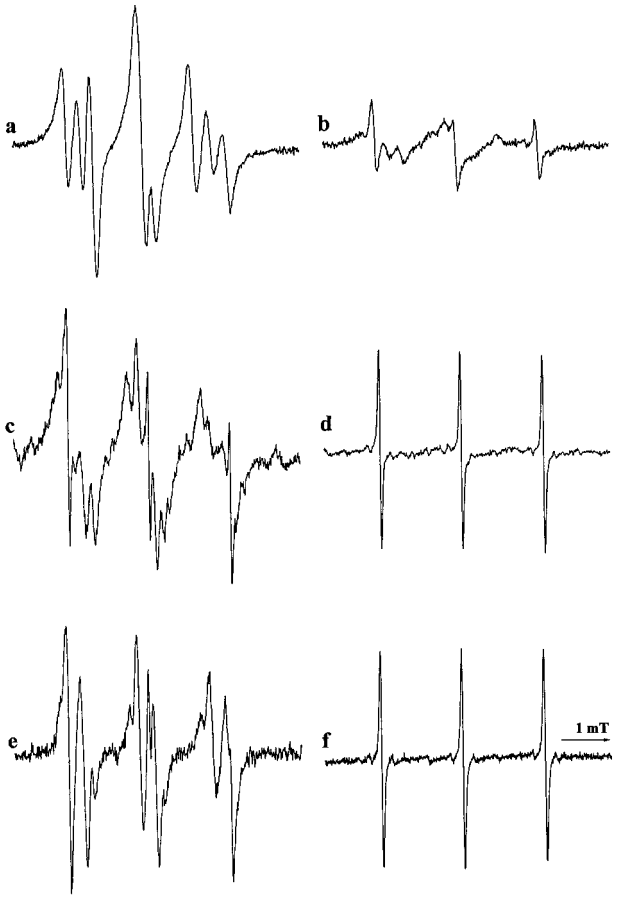


Figure 5 EPR spectra detected on reaction of histone H1-, melittin- or lysine-hydroperoxides with Cu^+, in the presence and absence of uridine and the spin trap MNP

Hydroperoxides were generated by ^{60}Co γ-irradiation in the presence of O_2 and assayed as reported in Figure 1 and the Materials and methods section. Reaction was initiated by addition of Cu^+ [formed *in situ* by the addition of deoxygenated solutions of Cu^{2+} (150 μM) followed by Ti^{3+} (100 μM)] to aqueous solutions of histone H1-, melittin- or lysine-hydroperoxides (typically 240, 50 and 38 μM, respectively) in the presence of the spin trap MNP (7.5 mM from a 100 mM stock in acetonitrile; final acetonitrile concentration 7.5% v/v) and uridine (saturated solution) at pH 7.4 and 20 °C. See the Materials and methods section for further experimental details. (**a**) Histone H1-OOH, (**b**) as (a) except in the absence of uridine. (**c**) Melittin-OOH, (**d**) as (c) except in the absence of uridine. (**e**) Lysine-OOH, (**f**) as (e) except in the absence of uridine. Typical EPR spectrometer settings: gain 1×10^6, modulation amplitude 0.025 mT, time constant 81 ms, scan time 83 s, centre field 347.5 mT, field scan 6 mT, with 8 scans accumulated. See text and Table 2 for details of assignments, hyperfine coupling constants, and relative radical adduct concentrations.

radical adduct on the basis of its low nitrogen coupling constant (approx. 1.047 mT); this species was also detected with uridine monophosphate, but not with uracil or 2′-deoxyuridine. The nature of this species has not been fully elucidated, but it may be a sugar-derived species whose formation requires the presence of the 2′-hydroxy group. In some cases weak signals from residual histone H1-hydroperoxide-derived radicals were also detected. No evidence was obtained for other sugar-derived species in accord with the selectivity observed with other radicals [25,34,35].

Studies with thymidine and cytidine also provided evidence for the formation of adducts between histone H1 hydroperoxide-derived radicals and pyrimidine bases, though in most cases the signals detected were less intense than those observed with uracil, uridine and related materials (data not shown). This may be due to steric interactions. As with uridine, little evidence was obtained for sugar radicals. Both the C_5-yl and C_6-yl uridine adducts were detected during the Cu^+-catalysed decomposition of both lysine and melittin hydroperoxides (Table 2). The spectra obtained with these hydroperoxides, in the presence of uridine, were weaker than those detected with histone H1-hydroperoxide, probably as a result of the lower concentration of hydroperoxides formed from these materials.

Formation of 8-oxodG as a result of oxidation of dG and DNA by protein- and amino acid-hydroperoxide-derived radicals

The above data are consistent with reaction of the hydroperoxide-derived radicals with DNA bases. To quantify such damage, and examine further the oxidation of the purine bases by such radicals, the formation and yield of 8-oxodG formed from both free 2′-deoxyguanosine (dG) and calf thymus DNA on Cu^+-catalysed breakdown of histone H1-, melittin- and lysine-hydroperoxides was examined. The yield of this product is expressed per parent dG present in order to compensate for any loss of material during isolation and purification (see the Materials and methods section).

Cu^+-catalysed decomposition of histone H1-, melittin- or lysine-hydroperoxides (approx. 280, 57 and 40 μM, respectively) in the presence of dG (200 μM) gave 8-oxodG (Figures 6 and 7). 8-OxodG was also detected when dG was replaced with calf thymus DNA, consistent with this lesion also being a product of DNA oxidation by protein- and amino acid-hydroperoxide-derived radicals. Other minor peaks were also detected by HPLC, particularly with DNA as the target; these presumably reflect other DNA oxidation products [36,37]. These were not characterized further. The 8-oxodG yield formed from dG or DNA increased with increasing concentrations of the starting protein- or amino acid-hydroperoxide (Figure 6), and was statistically significant ($P < 0.009$) when compared with that obtained from non-irradiated protein or Cu^+-free controls. The levels obtained in the latter experiments (incubation of hydroperoxides with target in the absence of Cu^+) were also consistently higher than with the non-irradiated protein, and the difference observed with histone H1-hydroperoxide was statistically significant with DNA ($P < 0.006$); that with free dG was not significant ($P < 0.094$). In the case of melittin- and lysine-hydroperoxides these values also reached statistical significance in most cases (Figure 7). The formation of 8-oxodG in the absence of added metal ions may be due to thermal decomposition of the hydroperoxides. The yield of 8-oxodG was reduced to background (non-irradiated protein) levels when irradiated histone H1, which had been previously treated with ebselen/GSH to reduce the hydroperoxide groups to the corresponding alcohols, was employed, demonstrating a requirement for hydroperoxide groups for the formation of 8-oxodG (data not shown).

The yield of 8-oxodG formed on oxidation of dG by histone H1-hydroperoxide in the presence of Cu^+ was found to be diminished when other nucleosides were included in the incubation mixture, demonstrating, as expected from the EPR data, that the histone H1-hydroperoxide-derived radicals also react with other DNA bases. Thus the level of 8-oxodG was significantly reduced (mean $\pm$ S.E.M., $n = 8$) in the presence of large excesses (100 mM) of 2′-deoxycytidine ($75.6 \pm 9.3\%$), thymidine ($97.6 \pm 1.5\%$) or 2′-deoxyuridine ($82.3 \pm 2.8\%$); all of these decreases were statistically significant ($P < 0.005$). Interestingly, when 2′-deoxyadenosine (saturated solution) was included in the reaction system, no inhibition of 8-oxodG formation was observed.

Table 2 EPR parameters of radical adducts detected during reaction of uridine with histone H1-, melittin- or lysine-hydroperoxides with Cu^+ in the presence of the spin trap MNP

See text and the Materials and methods section for further experimental details.

Substrate	Spin adduct assignment*	Hyperfine coupling constants (mT)†			Relative radical concentration (% of total)§
		a(*N*)	*a*(*H*)	*a*(*Other*) ‡	
Histone H1-hydroperoxide	Uridine C_5-yl	1.523	0.308		63 ± 12
	Uridine C_6-yl	1.469	0.206	0.262	14 ± 4
	Acyl‖	1.047			20 ± 8
Melittin-hydroperoxide	Uridine C_5-yl	1.638	0.402		11 ± 2
	Uridine C_6-yl	1.474	0.145	0.239	69 ± 1
Lysine-hydroperoxide	Uridine C_5-yl	1.519	0.319		40 ± 26
	Uridine C_6-yl	1.470	0.143	0.239	56 ± 15

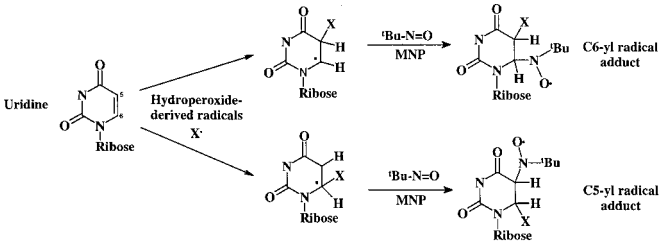
* See reaction 4 and text for nomenclature of spin adduct species.
† Typically ± 0.02 mT.
‡ Nitrogen of uridine ring at position 1.
§ Mean percentage values ($n = 18$ determinations, from computer simulations of experimental spectra, see the Materials and methods section) of total radical adduct concentration detected; values do not sum to 100% due to minor contributions from spin-trap degradation products.
‖ Possibly sugar derived; see text for further details.

DISCUSSION

Exposure of histone H1, melittin and lysine to γ-radiation in the presence of O_2 generates high yields of hydroperoxides in a dose-dependent manner; this is in accord with studies on other proteins and amino acids [5,6,8–10]. These hydroperoxides decompose slowly at 20 or 37 °C, in the absence of added metal ions, via complex kinetics; this is consistent with previous reports [5,18] and presumably reflects the heterogeneous nature of the hydroperoxides and their differing stabilities. These hydroperoxides decay rapidly when treated with Cu^+, and give rise to a number of carbon-centred radicals. These radicals are believed to arise via the formation of an initial (undetected) alkoxyl radical via a pseudo-Fenton reaction (reaction 1) and subsequent rearrangement/fragmentation (reaction 2; cf. studies with other proteins and amino acids [9,10]). Prior reduction of the histone H1-hydroperoxides to alcohols before reaction with Cu^+ resulted in the loss of these adducts, confirming that these species are hydroperoxide-derived. The exact sites of the precursor hydroperoxides, and hence the hydroperoxide-derived radicals, on the protein structure remain to be elucidated.

Previous studies have suggested that proteins are the most abundant target within cells for reaction with radicals such as $HO^{\bullet}$. Furthermore it is well established that within cell nuclei, histone and other nuclear proteins are major targets for radiation-induced damage. Thus irradiation of mixtures of isolated histone proteins results in almost identical levels (within 5%) of amino acid consumption to those observed with deoxyribonucleohistone complexes irradiated under identical conditions [38,39], implying that the majority of damage occurs on nuclear proteins, rather than DNA, under the conditions employed. Examination of the loss of individual amino acids in irradiated histone proteins shows that lysine residues are lost to a much higher extent [G approx. 0.31; where G is the radiation yield (number of species formed per 100 eV absorbed energy)] than all other residues ($G \leqslant 0.16$) [38,39]. Thus lysine residues, which comprise approx. 30 mol% of the amino acids present in histone H1, and which are known to give high yields of hydroperoxides when irradiated in free solution [6,8], are likely to be major sites of the hydroperoxides detected on irradiated histone H1. This conclusion is supported by product studies which have demonstrated the formation of significant yields of lysine hydroxides (at C-3, C-4 and C-5 on the side-chain) on histone H1 after γ-irradiation in the presence of O_2 [21].

The protein- and amino acid-hydroperoxide-derived radicals react readily with pyrimidine bases and nucleosides to give adduct species (i.e. protein/amino acid–DNA base cross-links); this is in accord with a recent report [23]. The nucleobase/nucleoside-derived radical adducts detected in the current study are believed to arise via the addition of a hydroperoxide-derived radical to the C_5–C_6 double bond of the pyrimidine ring, to give a hydroperoxide–nucleobase adduct, which then reacts with the spin trap to give the observed nitroxide adduct (cf. reaction 4).



(4)

Whether it is the primary alkoxyl radicals or the secondary carbon-centred radicals which react with the pyrimidine nucleobases/nucleosides/nucleotides is not clear at present. The predominance of attack at the C_6 position of uridine over C_5 (as judged by the approx. 3:1 ratio of the C_5-yl:C_6-yl adducts) is different from that previously reported for attack by electrophilic radicals such as $HO^{\bullet}$ and $^tBuO^{\bullet}$ as judged by both EPR [25,35] and pulse radiolysis [40,41] studies. This is consistent with the attacking radical being nucleophilic in nature (cf. studies with aryl radicals [42]) and therefore the secondary carbon-centred radicals formed as a result of rearrangement of the initial hydroperoxide-derived alkoxyl radical are likely candidates. Such reactions would result in carbon–carbon cross-links between the initial hydroperoxide-containing species and the base (cf. reaction 4, with $X^{\bullet}$ a carbon-centred radical). Steric factors may also, however, play a role in determining the selectivity of attack at C_5

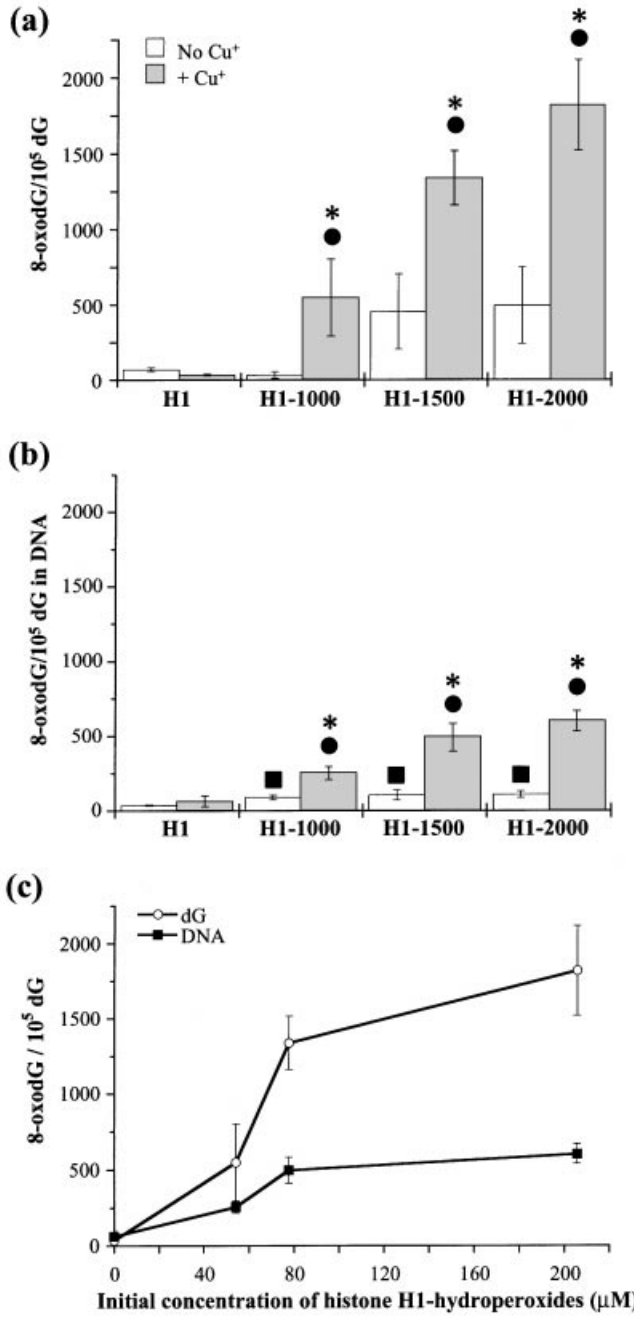


Figure 6 Formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) from dG (a) and calf thymus DNA (b) on reaction with increasing concentrations of histone H1-hydroperoxides in the presence of Cu^+

Histone H1 hydroperoxides were generated by ^{60}Co γ-irradiation (1000, 1500 or 2000 Gray total dose) in the presence of O_2 and assayed as reported in Figure 1 and the Materials and methods section. Reaction was initiated by addition of Cu^+ [formed *in situ* by the addition of deoxygenated solutions of Cu^{2+} (150 μM) followed by Ti^{3+} (100 μM)] to aqueous solutions of histone H1 hydroperoxides (280 μM) in the presence of 2′-deoxyguanosine (200 μM) at pH 7.4 and 20 °C. Samples subsequently incubated for 5 min, before reaction halted by addition of 1 volume of chloroform. Subsequent processing of samples and quantification of 8-oxodG was carried out as described in the Materials and methods section. (**c**) Plot of initial histone H1 hydroperoxide concentration versus yield of 8-oxodG. Values reported are means $\pm$ S.D. ($n = 4$ determinations). (●) Statistically elevated ($P < 0.05$) values compared with the non-irradiated (H1) Cu^+-treated controls. (*) Statistically elevated ($P < 0.05$) values compared with the corresponding irradiated samples incubated in the absence of Cu^+. (■) Statistically elevated ($P < 0.05$) values compared with the non-irradiated (H1) samples incubated in the absence of Cu^+.

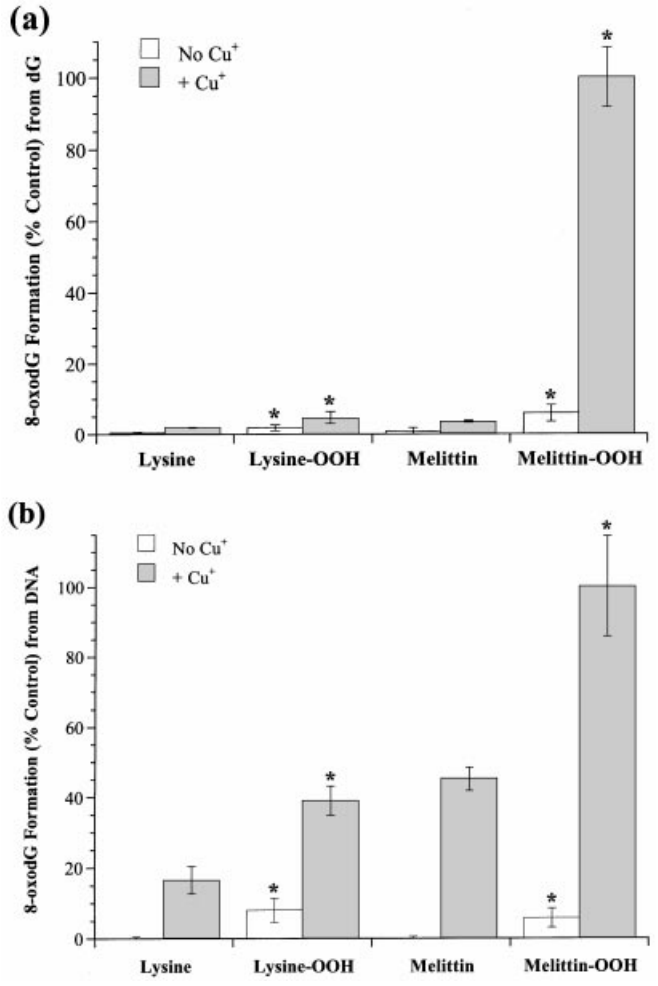


Figure 7 Formation of 8-oxodG from (a) dG and (b) calf thymus DNA after reaction with melittin- and lysine-hydroperoxides in the presence of Cu^+

Reactions and analyses were carried out as described in Figure 6 and the Materials and methods section, except using melittin- and lysine-hydroperoxides (57 and 40 μM, respectively). The values reported are percentages of the values obtained with melittin hydroperoxide plus Cu^+ in both cases, and are means $\pm$ S.E.M. ($n = 12$ determinations). (*) Values statistically significant relative to the corresponding non-irradiated sample ($P < 0.05$).

versus C_6 with the pyrimidine bases, particularly with the protein hydroperoxides.

EPR experiments using DMPO as the spin trap have provided evidence for reaction with purine bases, suggesting that these are also major targets; this is supported by the detection of 8-oxodG as a product. The yield of 8-oxodG has been shown to be proportional to the initial protein-hydroperoxide concentration, with only control levels detected with either non-irradiated, or irradiated and reduced, materials. Evidence has also been obtained from these products studies for reaction at cytosine, uridine and thymidine, but not adenosine; the lack of damage to the latter may result from rapid damage transfer within DNA to guanosine residues. The products arising from reaction at cytosine, uridine and thymidine have not been characterized further.

The formation of 8-oxodG could arise via reaction of the initial alkoxyl radical generated from these hydroperoxides, the carbon-centred radicals formed via their rearrangement/

fragmentation, or peroxyl radicals formed either as a result of molecular oxygen to these carbon-centred radicals, or via reaction 3. In this case, unlike the case with the pyrimidines, no stable adduct species (i.e. protein/amino acid bound to dG) has been detected by EPR, suggesting that the oxidizing species in this case is either the initial alkoxyl radical or peroxyl species; previous studies have shown that carbon-centred radicals react with purines via addition [24,43].

In recent studies we have demonstrated that 3,4-dihydroxyphenylalanine (DOPA; either free or protein-bound), another long-lived, reactive protein oxidation product formed by oxidation of Tyr residues, can also induce damage to DNA in the presence of copper ions [13]. Though this reaction is efficient, the concentration of tyrosine residues in histone H1 is very low [44], and hence the yield of protein-bound DOPA is very limited (estimated as $< 0.3\ \mu$M; data not shown), and should not contribute markedly to the reactions studied here. The potential for hydroperoxide-derived radicals to give DNA damage has been confirmed in experiments with irradiated melittin, which cannot give rise to DOPA as it contains no tyrosine residues [45], and lysine. The damage observed with irradiated melittin and lysine is therefore likely to be due solely to hydroperoxide-derived species. Our conclusion that damage observed with histone H1 also arises from hydroperoxide-derived radicals, and not DOPA, is supported by the absence of radical formation and DNA damage in samples which had been subjected to chemical reduction, a process which removes hydroperoxide functions but which does not reduce DOPA levels [13]. DOPA-mediated reactions, however, play a role in DNA damage induced by other radical-damaged nuclear proteins.

The yields of 8-oxodG obtained from both dG and DNA increase with the concentration of histone H1-hydroperoxide employed, though the overall extent of conversion into 8-oxodG is relatively small. Thus when approx. 280 μM histone hydroperoxide was reacted with dG (200 μM) or DNA (approx. 70 μg/ml) the conversion to 8-oxodG has been determined as approx. 2% (2000 molecules 8-oxodG per 10^5 dG) for free dG and 0.2% (600 molecules 8-oxoG per 10^5 dG) for dG in DNA, and therefore a yield of 8-oxodG per initial hydroperoxide group of approx. 1.4% for free dG and 0.14% for dG in DNA. These conversion yields are lower than those detected for free-, and protein-bound, DOPA, with dG and DNA, where the yield of 8-oxodG per initial DOPA is approx. 32% [13]; these values, however, do not take into account the relative yields of hydroperoxide versus DOPA groups on histone H1, where the former is orders of magnitude greater than the latter. Given that the initial conversion of $HO^{\bullet}$ into hydroperoxide groups with histone H1 can be approx. 40%, this gives an efficiency of 8-oxodG formation per initial $HO^{\bullet}$ of approx. 0.6% for free dG and 0.06% for dG in DNA, assuming all the hydroperoxide groups are consumed.

The lower yield of 8-oxodG formed from DNA, compared with free dG, is consistent with both the decreased yield of 8-oxodG formed from free dG in the presence of alternative nucleosides and the EPR data. The observation that excess 2′-deoxyadenosine had no effect on the yield of 8-oxodG generated from free dG is surprising; this may be because dG residues are more readily oxidized than 2′-deoxyadenosine, with the result that species formed on oxidation of 2′-deoxyadenosine might subsequently transfer damage to the dG. This is also consistent with reaction of the hydroperoxide-derived species with the purine bases being an oxidation rather than addition reaction. The transfer of (positive hole) damage from other bases to guanosine residues has been widely documented in radiation studies on DNA (e.g. [46]).

The occurrence of similar damage transfer reactions from other histone- and non-histone nuclear protein-hydroperoxide-derived radicls to isolated DNA, and within intact nucleohistone particles, remains to be established; preliminary studies suggest that such processes also occur (C. Luxford and M. J. Davies, unpublished data). Whether such reactions can also occur in the absence of added transition metal ions such as Cu^{+}, i.e. whether such reactions are catalysed by endogenous nuclear metal ions, remains to be investigated. Previous studies, however, have reported the presence of redox-active transition metals in the nucleus (e.g. Cu^{2+} bound to DNA or nuclear matrix proteins [11,12,47,48]). However, some of the results obtained in this study show that the presence of such transition metal ions might not be an absolute pre-requisite for DNA oxidation. Thus it has been shown that the amino acid- and protein-hydroperoxides undergo (spontaneous) decomposition at 20 and 37 °C, and that the yields of 8-oxodG formed on incubation of these hydroperoxides with both free dG, and dG in DNA, are in some cases elevated statistically compared with the corresponding non-irradiated samples. This finding suggests that slow thermal decomposition of these hydroperoxides, in the absence of added metal ions, can also initiate conversion of both free dG, and dG in DNA, to the mutagenic lesion 8-oxodG. This process may be important *in vivo*, if the protein hydroperoxides are not rapidly repaired.

Thus the generation of radicals from protein hydroperoxides formed on nuclear proteins as a result of initial radical-induced damage to the protein component of nuclei, in the presence of O_2, can provide a novel route to the formation of both mutagenic (8-oxodG) lesions in DNA, and protein-DNA crosslinks (see also [11,12,23,47,48]). The reactions described in this study and elsewhere [11,12,23,47,48], may therefore play an important role in radical-induced nuclear damage and carcinogenesis.

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