The protein oxidation product 3,4-dihydroxyphenylalanine (DOPA) mediates oxidative DNA damage

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A major product of hydroxy-radical addition to tyrosine is 3,4-dihydroxyphenylalanine (DOPA) which has reducing properties. Protein-bound DOPA (PB-DOPA) has been shown to be a major component of the stable reducing species formed during protein oxidation under several conditions. The aim of the present work was to investigate whether DOPA, and especially PB-DOPA, can mediate oxidative damage to DNA. We chose to generate PB-DOPA using mushroom tyrosinase, which catalyses the hydroxylation of tyrosine residues in protein. This permitted us to study the reactions of PB-DOPA in the virtual absence of other protein-bound oxidation products. The formation of two oxidation products of DNA, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodG) and 5-hydroxy-2′-deoxycytidine (5OHdC), were studied with a novel HPLC using gradient elution and an electrochemical detection method, which allowed the detection of both DNA modifications in a single experiment. We found that exposure of calf thymus DNA to DOPA or PB-DOPA resulted in the formation of 8oxodG and 5OHdC, with the former predominating. The formation of these DNA oxidation products by either DOPA or PB-DOPA depended on the presence of oxygen, and also on the presence and on the concentration of transition metal ions, with copper being more effective than iron. The yields of 8oxodG and 5OHdC increased with DOPA concentration in proteins. Thus PB-DOPA was able to promote further radical-generating events, which then transferred damage to other biomolecules such as DNA.

INTRODUCTION

Radical-induced damage to proteins has usually been thought to produce relatively inert chemical species by cross-linking or fragmenting proteins, or by chemical modification of amino-acid side chains. These alterations often result in changes in structure and conformation and loss of enzymatic activity [1–3]. However, it has been shown recently that two products of protein oxidation, i.e. protein-bound reducing moieties, comprising mainly 3,4-dihydroxyphenylalanine (DOPA), [4–6] and protein hydroperoxides [5,7–9], are able to initiate further chemical reactions. Thus protein- or peptide-bound DOPA (PB-DOPA) has been shown to reduce cytochrome *c*, free Fe and Cu ions [4–6], and the protein hydroperoxides have been shown to destroy some antioxidants *in vitro* [9,10] and to give rise to reactive radicals [11,12]. PB-DOPA can be generated on a number of different proteins by exposure to both radicals generated by γ-radiolysis, UV irradiation, Fenton-type systems [5] and the enzyme tyrosinase [6,13]. PB-DOPA has also been detected *in vivo*, for example, on intimal proteins from human atherosclerotic plaques and in cataractous lens proteins [14], and this has been postulated as a marker of radical-induced protein damage. We have proposed that this protein-bound moiety might play a significant role in the propagation of pathological radical damage to other cellular components such as DNA [15]. In agreement with this idea, it has been reported recently that free L-DOPA, a metabolite in the nervous system, in the presence of Cu(II) and H_2O_2 leads to extensive DNA damage [16] and can cause strand breakage in DNA *in vitro*, even in the presence of Cu(II) alone [17]. Whenever PB-DOPA is formed on proteins, it may thus undergo redox interconversions between catechol and quinone forms, and further oxidation to give additional catechol–quinone systems [4,13]; in the remainder of this paper these forms will be referred to generically as PB-DOPA.

8-Oxo-7,8-dihydroxy-2′-deoxyguanosine (8oxodG) can be generated from 2′-deoxyguanosine (dG) in DNA by the attack of hydroxy radicals [18,19], by one electron oxidation [20] and by exposure to singlet oxygen [21,22]. It has been used widely as an indicator of oxidative damage in DNA and as a general marker of oxidative stress [23–25]. Similarly, 5-hydroxy-2′-deoxycytidine (5OHdC) is one of the major products of the OH radical-induced decomposition of 2′-deoxycytidine (dC) in DNA [26]. Thus determination of 8oxodG and 5OHdC formation in DNA after reaction with PB-DOPA provides a sensitive method of assessing the potential transfer of damage from oxidized proteins to DNA.

In this study, 8oxodG and 5OHdC in DNA were measured using HPLC with electrochemical detection (EC). For this purpose, we have developed a new HPLC–EC method using gradient elution, allowing the detection of both DNA-modification products in a single run, with a sensitivity of about 500 fmol for each. 8oxodG and 5OHdC formation in DNA as a result of exposure to both free DOPA and PB-DOPA (insulin, BSA, Gly-Tyr-Gly) was examined in the presence (and absence) of oxygen and exogenous transition metal ions. The results indicated that PB-DOPA was capable of catalysing the generation of further radicals, which cause damage to DNA.

MATERIALS AND METHODS

Materials

dG, dC, thymidine, calf thymus DNA, L-DOPA, mushroom tyrosinase, fatty-acid free BSA, melittin, glucose oxidase and catalase were purchased from Sigma (St. Louis, MO, U.S.A.). Bovine pancreas insulin, nuclease P1 and alkaline phosphatase

Abbreviations used: dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; DOPA, 3,4-dihydroxyphenylalanine; PB-DOPA, protein- or peptide-bound DOPA; GYG-DOPA, Gly-Tyr-Gly-bound DOPA; BSA-DOPA, BSA-bound DOPA; Ins-DOPA, insulin-bound DOPA; ED, ethylenediamine; Ins-DOPA-ED, insulin-bound DOPA derivatized with ethylenediamine; BSA-DOPA-ED, bovine-serum-albumin-bound DOPA derivatized with ethylenediamine; 8oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 5OHdC, 5-hydroxy-2′-deoxycytidine; EC, electrochemical detection.

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were provided by Boehringer (Mannheim, Germany). 3-(2,4-Dihydroxyphenyl)propionic acid was purchased from Fluka (Buchs, Switzerland). The tripeptide, Gly-Tyr-Gly (GYG), was obtained from Auspep (Parkville, Victoria, Australia). Ethylenediamine (ED) and ethylenediamine dihydrochloride were provided by Aldrich (Milwaukee, WI, U.S.A.). Chelex-100 resin was supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other materials were of analytical grade and were from Merck (Darmstadt, Germany) and BDH (Kilsyth, Victoria, Australia). Water used was purified by passage through a four-stage Milli-Q system (Millipore–Waters, Australia). HPLC-grade methanol was from Mallinckrodt (St. Louis, MO, U.S.A.). 8oxodG and 5OHdC standards were kindly given by Dr. Jean Cadet (Centre d'Etudes Atomiques, Grenoble, France).

Preparation of PB-DOPA

Incubation of proteins with tyrosinase

As described by Ito et al. [13], BSA (64 mg) and insulin (64 mg) were incubated at 30 °C with mushroom tyrosinase (3 mg, 8300 units/mg; 1 unit causes an increase in A_{280} of 0.001/min at pH 6.5 at 25 °C in a 3 ml reaction mixture containing L-tyrosine) in 64 ml of sodium phosphate buffer (100 mM, pH 7.4). At various time points, the reaction was stopped by the addition of 16 ml trichloroacetic acid (10 % w/v). The highest formation of DOPA in proteins was found after 6 h incubation (51 pmol DOPA/nmol of insulin [4]; 31 pmol DOPA/nmol of BSA). The protein was subsequently pelleted by centrifugation (at 735 ***g*** for 35 min) after being kept overnight at 4 °C, redissolved in 20 ml of water and stored at −20 °C until used.

γ-Irradiation of proteins

Insulin solutions (2 mg/ml) were irradiated in the presence of oxygen using a ^{60}Co source to a total dose of 960 Gy. This lead to the formation of 75 pmol DOPA/nmol of insulin as shown previously [4]. After irradiation the solutions were stored at −20 °C until used.

Determination of the amount of DOPA in PB-DOPA

Gas-phase amino-acid hydrolysis of protein

Protein solution (200–300 μg of protein in 100 μl was placed in a 0.7 ml glass auto-sampler vial (Alltech) and freeze-dried in a vacuum centrifuge. Sample vials were then placed in a Pico-Tag reaction vessel (Millipore–Waters, Australia) to which 1 ml of 6 M HCl containing 1 % (w/v) phenol and 50 μl of mercaptoacetic acid were added. After thorough deoxygenation with N_2, the reaction vessel was evacuated and heated to 110 °C for 17 h. After hydrolysis, residual acid was removed by vacuum centrifugation and the hydrolysate was dissolved in water for HPLC analysis.

HPLC analysis of protein-acid hydrolysate for DOPA

DOPA yields were assessed by chromatography on a Zorbax ODS column (4.6 mm × 25 cm; Rockland Technologies Inc. (Newport, DE, U.S.A.) as described previously [27]. A gradient of buffer A (100 mM sodium perchlorate/10 mM sodium phosphate, pH 2.5) in solvent B (80 % methanol in water) was used with a flow rate of 1 ml/min. The gradient profile was as follows: isocratic elution with 0 % solvent B for 12 min; a gradient of 20 % solvent B in 8 min; further elution at 20 % solvent B for 3 min before a gradient to 50 % solvent B in 3 min; isocratic elution at 50 % solvent B for another 3 min and finally, re-equilibration with 100 % buffer A for 10 min. The eluent was monitored by UV (Shimadzu) at 280 nm and fluorescence (Hitachi F-1080) detectors in series. Fluorescence excitation was at 280 nm and emission was monitored at 320 nm. Peak positions and quantification were defined on the basis of standards. Protein recovery was assessed by UV measurement of *p*-tyrosine residues.

Reaction of DNA with DOPA or PB-DOPA

Standard reaction mixtures (1 ml) contained calf thymus DNA (200 μg; pre-treated with Chelex resin to remove the metal ions) insulin-bound DOPA (Ins-DOPA) (100 μM insulin, 4.5 μM DOPA) or BSA-bound DOPA (BSA-DOPA) (20 μM BSA, 0.5 μM DOPA) or DOPA (10 μM) and $CuSO_4$ or $FeCl_3$ (metal ion/protein molar ratio, 5:1, as shown in Figure 2 and as discussed in the Results section). Reagent concentrations for individual experiments are given in the Figure legends. Reactions were started by adding the metal ion and vortex mixing.

For anaerobic experiments, two different conditions were used. The anoxic incubation was established by bubbling 1 ml of solution containing 200 μg DNA, 100 μM DOPA and 500 μM $CuSO_4$ with N_2 for 15 min in a septum-sealed vial. The controls were not degassed with N_2. The anaerobic incubations were similar except that the solutions also contained glucose oxidase (250 units) and glucose (60 mM), as described by Kelman and Mason [28]. H_2O_2 was generated in this system, therefore, catalase (10 μg/ml) was added. Control incubations were bubbled with a continuous flow of air to ensure that the solution was saturated with oxygen.

The samples were incubated at 37 °C for 1 h and the reactions were terminated by adding 100 μl of 3 M sodium acetate and two volumes of cold ethanol (−20 °C). Solutions were kept overnight at −20 °C and DNA was subsequently recovered by centrifugation at 735 ***g*** for 40 min. Blanks were prepared with protein which had not been treated with tyrosinase, or without metal ion, or without PB-DOPA.

Hydrolysis of isolated DNA

DNA pellets were incubated in 200 μl of water with 21 μl of nuclease P1 buffer [300 mM sodium acetate (pH 5.3)/1 mM $ZnSO_4$] and 10 μl (10 units) of nuclease P1 solution. Samples were then incubated at 37 °C for 2 h. Dephosphorylation of the resulting nucleotides was achieved by the addition of 23 μl of 500 mM Tris-HCl, pH 8.0/1 mM EDTA buffer and 1 μl (1 unit) of alkaline phosphatase solution. After incubation for 1 h at 37 °C, the proteins were precipitated by the addition of 100 μl of chloroform. Samples were then centrifuged and the aqueous layers collected. The remaining proteins in the hydrolysates were removed using a Microcon-30 microconcentrator (30000 molecular-mass cut-off) (Amicon, Beverly, U.S.A.) and then concentrated to dryness. The samples were dissolved in 100 μl of water before HPLC analysis.

HPLC–EC analysis

The HPLC system consisted of a SIL-10A auto injector (Shimadzu, Kyoto, Japan), two LC-10AT pumps (Shimadzu, Kyoto, Japan), a 5100A Coulochem II electrochemical detector (ESA, Chelmsford, MA, U.S.A.) and an SPD-10A UV detector (Shimadzu, Kyoto, Japan). The data was digitised using a CBM-10A interface (Shimadzu, Kyoto, Japan) and processed on an IBM PC 123 computer.

8oxodG and 5OHdC were separated by HPLC using a Zorbax ODS column (4.6 mm × 25 cm; Rockland Technologies Inc.) at

a flow rate of 1 ml/min, eluted with a gradient of buffer A (50 mM potassium phosphate, pH 5.5) in solvent B (methanol/water, 80:20, v/v). The gradient was generated as follows: 5 min at 100 % buffer A, a gradient to 21 % solvent B in 16 min, 100 % buffer A continuously until 40 min. Detection potentials were set at +50 mV and +400 mV for electrodes 1 and 2 respectively. Peak identification and calibration were carried out with pure 5OHdC and 8oxodG and the retention times of 5OHdC and 8oxodG were 7.1 and 18.1 min respectively. These conditions allowed detection of about 500 fmol of each oxidized nucleoside. Unmodified nucleosides were monitored by UV detection at 254 nm. The amount of DNA analysed was calculated from the area of the peak for thymidine after appropriate calibration.

ED derivatization

ED derivatization was performed as described previously [29], by adding 2 M ED dihydrochloride (100 μl) and ED (140 μl), to bring the mixture to pH 10.4, to a known volume of sample diluted to 2 ml with 5 mM phosphate buffer, pH 7.4. The mixture was briefly vortex-mixed and heated at 50 °C in a water-bath for 120 min. The protein solution was then applied to a PD-10 column to remove excess ED, which may interfere during the incubation of DNA and PB-DOPA.

EPR spectroscopy

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation. Samples were contained in a standard, flattened aqueous sample cell and the recording of spectra was usually initiated within 2 min of the start of the reaction. EPR spectrometer setting were: gain 5×10^4, modulation amplitude 0.2 mT, time constant 0.32 s, conversion time 0.08 s, scan time 168 s, resolution 2048 points, centre field 320 mT, field scan 80 mT, power 25 mW, frequency 9.74 gHz, 1 scan.

RESULTS

Analysis of 8oxodG and 5OHdC formation in DNA

An HPLC–EC method was developed to detect both 8oxodG and 5OHdC in a single HPLC run. For this purpose, gradient elution, which is not commonly used with EC detection, was employed (Figure 1). The first EC well was set at +50 mV with the detection of 8oxodG and 5OHdC achieved in the second cell set at +400 mV. 5OHdC was slightly oxidized in the first EC cell but most of it was oxidized by the second electrode. The addition of methanol during the HPLC run induced an off-scale response of the baseline between 11 and 14 min, but this did not affect 8oxodG detection at 18.1 min. The sensitivity limits for both 8oxodG and 5OHdC were approx. 500 fmol. Consequently, no detectable 5OHdC was observed when 50 μg DNA were injected, corresponding to a modification rate of < 1 5OHdC/10^5 dC molecules. In contrast, 8oxodG was detected in hydrolysed DNA at a level of 5 8oxodG/10^5 dG molecules. Both materials were detected with calf thymus DNA incubated with Cu(II) alone, but in very low quantities (~ 15 8oxodG/10^5 dG and 1 5OHdC/10^5 dC molecules).

Formation of 8oxodG and 5OHdC in calf thymus DNA by Cu(II) in the presence of DOPA or PB-DOPA

Exposure of dG, dC or calf thymus DNA to DOPA, GYG-bound DOPA (GYG-DOPA), Ins-DOPA or BSA-DOPA hardly increased oxidative base damage over that already present in the commercial material (Table 1 and Table 2). Addition of copper ions in the absence of DOPA or PB-DOPA caused a small increase in the yield of 8oxodG and 5OHdC [24]. However, when DOPA [16] or PB-DOPA were incubated with dG, dC or DNA and Cu(II) there was substantial formation of 8oxodG and 5OHdC. Addition of 5 μM DOPA or PB-DOPA to DNA–Cu(II) system caused a ten-fold increase in 8oxodG formation and a

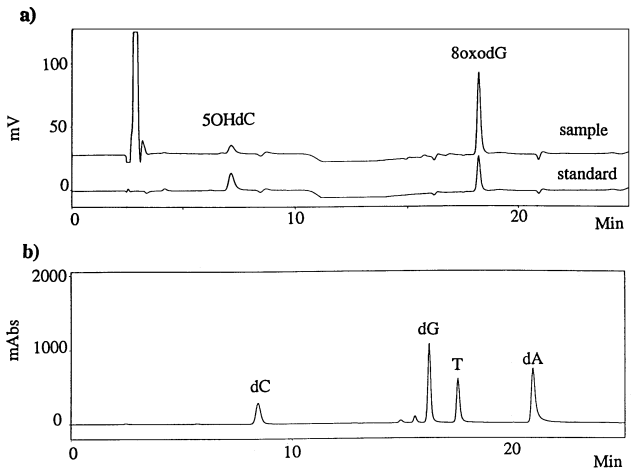

Figure 1 HPLC profile of a DNA sample treated with Cu(II) and Ins-DOPA

Production of 8oxodG and 5OHdC by Cu(II) and Ins-DOPA. DNA was incubated with Ins-DOPA and hydrolysed enzymically as described in the Materials and methods section. The hydrolysate was then analysed by HPLC with simultaneous 254 nm absorbance (**b**) and EC (**a**). 8oxodG and 5OHdC (6.25 pmol) (bottom trace in **a**) were used as standards.

Table 1 Damage to DNA by Cu(II) in the presence of DOPA, GYG-DOPA and BSA-DOPA

Reaction mixtures contained dG (200 μM), dC (200 μM) or calf-thymus DNA (200 μg), $CuSO_4$ (100 μM) and DOPA (0.5 μM), GYG-DOPA (0.5 μM DOPA, 4.5 μM GYG) or BSA-DOPA (0.5 μM DOPA, 20 μM BSA). After the reaction was terminated, the DNA pellet was digested enzymically and analysed by HPLC–EC (see the Materials and methods section). The results are the means ± range of two independent experiments. * Values below the detection limit.

		8oxodG/10^5dG	5OHdC/10^5dC
dG +		*	
	Cu(II)	5.0 ± 1.3	
	DOPA	4.3 ± 0.1	
	Cu(II) + DOPA	32.2 ± 8.7	
dC +			*
	Cu(II)		*
	DOPA		1.2 ± 0.1
	Cu(II) + DOPA		4.1 ± 0.8
DNA +		3.8 ± 0.3	*
	Cu(II)	10.7 ± 1.4	0.6 ± 0.6
	DOPA	4.0 ± 2.3	*
	Cu(II) + DOPA	34.6 ± 1.4	4.6 ± 0.4
	GYG-DOPA	3.2 ± 2.7	*
	Cu(II) + GYG	14.2 ± 1.4	1.1 ± 0.2
	Cu(II) + GYG-DOPA	67.5 ± 7.8	2.5 ± 2.7
	BSA-DOPA	13.0 ± 2.9	0.4 ± 0.7
	Cu(II) + BSA	34.2 ± 15.3	*
	Cu(II) + BSA-DOPA	71.6 ± 1.2	14.4 ± 0.9

Table 2 Damage to DNA by Cu(II) in the presence of DOPA, GYG-DOPA and Ins-DOPA

Reaction mixtures contained dG (200 μM), dC (200 μM) or calf-thymus DNA (200 μg), $CuSO_4$ (500 μM) and DOPA (5 μM), 3-(2,4-dihydroxyphenyl)propionic acid (100 uM), GYG-DOPA (5 μM DOPA, 45 μM GYG) and Ins-DOPA (5 μM DOPA, 100 μM insulin). After termination of the reaction, the DNA pellet was digested enzymically and analysed by HPLC–EC (see the Materials and methods section). Results are means ± range of two independent experiments. * Values below the detection limit.

		8oxodG/10^5dG	5OHdC/10^5dC
dG +		*	
	Cu(II)	10.5 ± 1.6	
	DOPA	0.8 ± 1.6	
	Cu(II) + DOPA	144.0 ± 7.7	
dC +			*
	Cu(II)		0.4 ± 0.1
	DOPA		4.4 ± 1.7
	Cu(II) + DOPA		7.8 ± 0.8
DNA +		3.2 ± 0.3	*
	Cu(II)	22.6 ± 2.1	1.7 ± 0.3
	DOPA	3.1 ± 0.1	*
	Cu(II) + DOPA	223.7 ± 30.5	11.5 ± 2.5
	3-(2,4-dihydroxyphenyl)propionic acid	7.2 ± 0.4	*
	GYG-DOPA	3.7 ± 0.8	*
	Cu(II) + GYG	40.1 ± 0.1	1.6 ± 0.9
	Cu(II) + GYG-DOPA	297.4 ± 66.0	5.1 ± 0.7
	Ins-DOPA	6.5 ± 1.2	*
	Cu(II) + Ins	15.28 ± 7.5	2.3 ± 2.5
	Cu(II) + Ins-DOPA	243.3 ± 19.7	6.0 ± 1.0

Table 3 Chemical deactivation of DOPA sites in proteins by ED derivatization

Ins-DOPA and BSA-DOPA were derivatized by ED as described in the Materials and methods section. After derivatization, the samples were applied to a PD 10 column. The reaction with DNA was then performed as described in the Materials and methods section with the following final reactant concentration: calf-thymus DNA (200 μg), Ins-DOPA and Ins-DOPA-ED (5.6 μM DOPA, 45 μM insulin), BSA-DOPA and BSA-DOPA-ED (0.4 μM DOPA, 13 μM BSA), and $CuSO_4$ (500 μM) for insulin samples and $CuSO_4$ (100 μM) for BSA samples. Positive controls included PB-DOPA which were applied to a PD-10 column but were not derivatised by ED and the addition of fresh PB-DOPA to a PB-DOPA-ED solution. Melittin was incubated with tyrosinase for 6 h as described in the Materials and methods section to give tyrosinase-treated melittin. Protein concentration was determined using bicinchoninic acid method (Sigma) with BSA as standard. Melittin and tyrosinase-treated melittin (80 μM) were incubated with DNA (200 μg) and $CuSO_4$ (500 μM). Results are means ± range of two independent experiments.

		8oxodG/10^5dG
DNA + Cu(II) (500 μM)		24.1 ± 0.9
	+ Ins-DOPA	144.7 ± 11.8
	+ Ins-DOPA-ED	29.5 ± 5.0
	+ Ins-DOPA-ED + fresh Ins-DOPA	96.4 ± 10.5
	+ Tyrosinase-treated melittin	25.7 ± 1.9
	+ Melittin	15.3 ± 7.8
DNA + Cu(II) (100 μM)		12.6 ± 1.3
	+ BSA-DOPA	42.2 ± 1.4
	+ BSA-DOPA-ED	5.7 ± 0.2
	+ BSA-DOPA-ED + fresh BSA-DOPA	35.6 ± 2.8

five-fold increase in 5OHdC formation (Table 2). Insulin which had not been exposed to tyrosinase, when incubated with DNA and Cu(II) had a minor inhibitory effect on 8oxodG and 5OHdC formation; this may be due either to binding of the Cu^{2+} ions to the insulin or to the protein acting as a radical scavenger. In contrast, BSA–Cu(II) added to DNA had the opposite effect; this was probably due to the presence of free reducing groups, such as thiols [5] and also of significant amounts of DOPA present in commercial BSA [13] (8 pmol DOPA/nmol of BSA, i.e. approx. 4 DOPA/10^3 tyrosine molecules, which contributes 0.15 μM DOPA to our reaction system). This concentration is in agreement with the concentration of DOPA found in plasma proteins (6 DOPA/10^3 tyrosine molecules [14]. Substantial DNA damage was seen at DOPA concentrations as low as 0.5 μM (Table 1).

DOPA in tyrosine-treated protein is the reactive species in the propagation of damage to DNA

ED derivatization blocks DNA damage

To determine if DOPA was essential for the transfer of damage to DNA or whether other species, such as tyrosinase or other protein-bound reducing species, were involved, PB-DOPA was treated with ED to selectively derivatize (deactivate) DOPA [29]. ED derivatization is selective for *o*-diphenol (catechol) moieties [30] and involves condensation between ED and the two carbonyl groups of the *o*-benzoquinones, derived from their oxidation. Excess ED was removed from the reaction mixtures, as this might interfere during the reaction with DNA. Neither Ins-DOPA nor BSA-DOPA derivatized with ED gave rise to the formation of 8oxodG on incubation with DNA (Table 3). Furthermore, the addition of fresh PB-DOPA to the PB-DOPA derivatized by ED almost restored 8oxodG formation in DNA (Table 3). Hence, the possible inhibitory role played by the remaining ED or PB-DOPA-ED in the reaction can be discounted.

A tyrosine-free protein (melittin), when reacted with tyrosinase, does not induce DNA damage

Melittin, a tyrosine-free oligopeptide, was treated with tyrosinase as described in the Materials and methods section for insulin and BSA. 8oxodG formation in DNA was not enhanced by reaction with either Cu^{2+}–melittin or Cu^{2+}–tyrosinase-treated melittin in comparison with that detected in samples reacted with Cu^{2+} alone (Table 3).

Taken together, these experiments confirm that PB-DOPA is a key component in damage transfer to DNA.

Reaction requirements for 8oxodG and 5OHdC generation by Cu(II) + PB-DOPA

Dependence of the reaction on Cu(II): protein ratio

It is well known that proteins (and particularly BSA) can bind copper ions tightly to specific sites, some of which are less available for redox reactions [31]. The dependence of 8oxodG and 5OHdC formation on Cu(II): protein ratio was investigated to evaluate the optimum Cu(II) concentration in promoting oxidative DNA damage (Figure 2). 8oxodG and 5OHdC production was significant even at a low copper to protein ratio (1:1). Maximum formation of 8oxodG and 5OHdC was observed at a 10:1 ratio of copper to protein. No 8oxodG or 5OHdC production was seen with Ins-DOPA in the absence of Cu(II), confirming a direct role for this metal ion in promoting damage to DNA. BSA-DOPA showed different behaviour, with a significant formation of 8oxodG and 5OHdC without the addition of Cu^{2+} ions, indicating the probable presence of contaminating

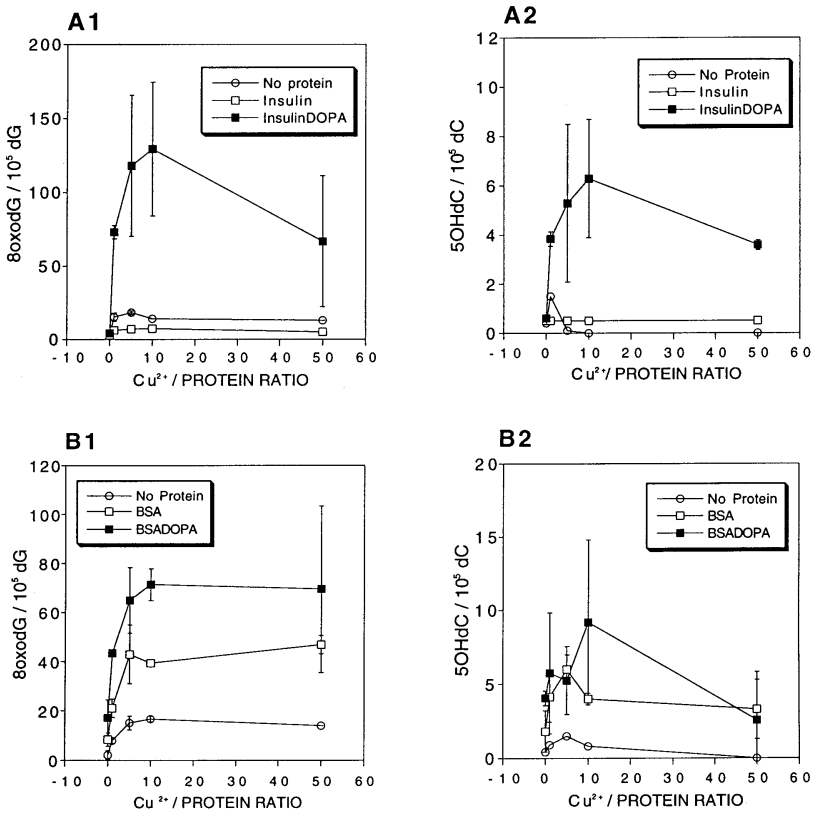

Figure 2 Effect of increasing Cu(II)/protein ratio on 8oxodG and 5OHdC concentration in DNA exposed to PB-DOPA

Ins-DOPA (5 μM DOPA, 100 μM insulin) or BSA-DOPA (0.5 μM DOPA, 20 μM BSA) were incubated with 200 μg calf-thymus DNA and increasing amount of $CuSO_4$ (■). (**A1**), 8oxodG formation with Ins-DOPA; (**A2**), 5OHdC formation with Ins-DOPA; (**B1**), 8oxodG formation with BSA-DOPA; (**B2**), 5OHdC formation with BSA-DOPA. Controls included DNA treated with $CuSO_4$ (○) and $CuSO_4$ + native protein (□). The results are the means ± range of two independent experiments.

protein–bound transition metal ions at a concentration sufficient to cause some oxidative modification to DNA.

Dependence of the reaction on DOPA concentration

Figure 3 (A1) and (A2) show the effect on the production of 8oxodG and 5OHdC by varying the amount of free DOPA in the DNA–Cu(II) system. Even very low concentrations of DOPA (0.5 μM) in the DNA/Cu(II) solution generated substantial yields of 8oxodG and 5OHdC.

Analogous experiments were performed with BSA- and Ins-DOPA (Figures 3, B1, B2, C1 and C2). The amount of protein used in these experiments was kept constant, so that the DOPA concentration in proteins was the only variable in the system. A 12-fold increase in 8oxodG formation was observed in increasing the DOPA concentration in insulin from 0.1 μM to 5 μM. For BSA, the range of achievable DOPA concentrations was small (0.12 μM–0.76 μM) and only relatively minor increases were expected. Nevertheless, enhanced 8oxodG and 5OHdC yields were clearly obtained with the higher concentrations of BSA-DOPA (Figures 3, C1 and C2).

Time course of 8oxodG formation

A kinetic study of 8oxodG formation by Ins-DOPA–Cu(II) is presented in Figure 4. The results indicated a fast formation of 8oxodG during the first hour with slower subsequent production. No substantial degradation of 8oxodG (98 %) was found when 8oxodG (2 μM) was incubated with Cu(II) (500 μM) for 22 h, indicating that destruction of the 8oxodG formed in the reaction was not occurring. The reaction did not reach completion even after 24 h of incubation. This is not surprising as the molar ratio of DOPA supplied to the 8oxodG formed was relatively high by this stage.

Role of Fe(III) on 8oxodG formation

Fe(III) was able to promote some DNA damage in presence of DOPA, BSA-DOPA and Ins-DOPA but the stimulation of damage by this metal ion was not as dramatic as with Cu(II), in agreement with previous findings [16] (Table 4).

Involvement of O_2 in the reaction

Experiments were performed to determine the effects of O_2 on the DOPA–Cu(II)–DNA system (results not shown). In all cases, inhibition of 8oxodG formation was observed and the level of inhibition was dependent on the specific means of deoxygenation. Bubbling of N_2 through the system resulted in 23–46 % inhibition, whereas use of both N_2 bubbling (15 min) together with a glucose–glucose oxidase system [28] (with added catalase to remove H_2O_2 generated by this system) resulted in 60 % inhibition. We conclude from these experiments that the reaction is O_2 dependent and our inability to obtain complete inhibition is a reflection of the difficulty in achieving complete O_2 removal.

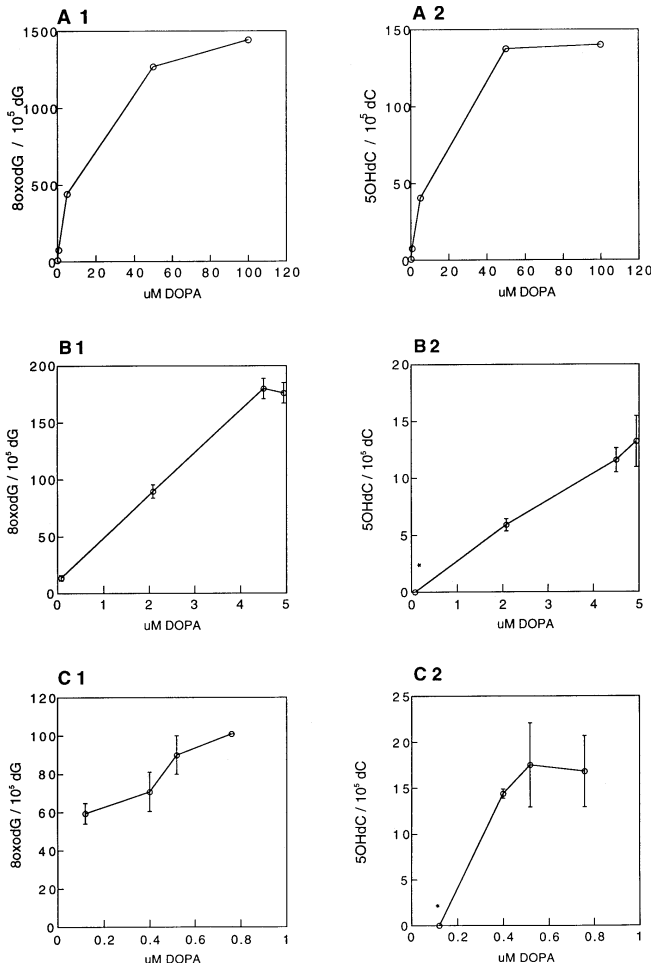

Figure 3 8oxodG and 5OHdC formation in DNA are dependent on DOPA concentration

(**A1**) 8oxodG and (**A2**) 5OHdC formation in DNA after the reaction of 200 μg calf-thymus DNA with 200 μM $CuSO_4$ and increasing amounts of DOPA. (**B1**) 8oxodG and (**B2**) 5OHdC formation in DNA after incubation of 200 μg calf-thymus DNA with 500 μM $CuSO_4$ and increasing amounts of DOPA in Ins-DOPA (100 μM insulin). (**C1**) 8oxodG and (**C2**) 5OHdC in DNA after incubation of 200 μg calf-thymus DNA with 100 μM $CuSO_4$ and increasing amounts of DOPA in BSA-DOPA (20 μM BSA). DOPA was present at the final concentrations indicated. The values in (**B1**), (**B2**), (**C1**) and (**C2**) are the means $\pm$ S.D. of three independent experiments. * Values below the detection limit.

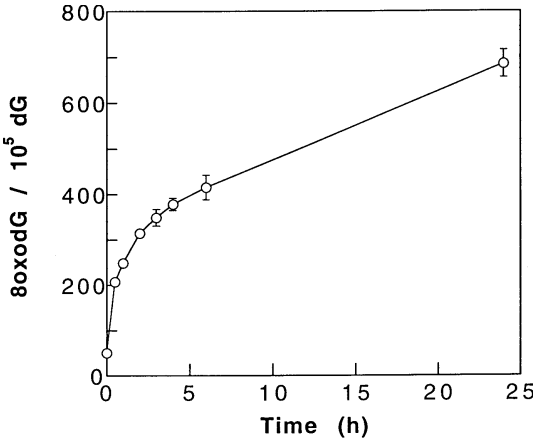

Figure 4 Time course of 8oxodG formation by Ins-DOPA–Cu(II) system

Samples (1 ml) containing 200 μg of calf-thymus DNA, $CuSO_4$ (500 μM) and Ins-DOPA (9 μM DOPA, 50 μM insulin) were incubated at 37 °C. The reaction was stopped by the addition of 2 ml of ice-cold ethanol and 100 μl of sodium acetate (3 M) at various time intervals. Results are the means $\pm$ range of two independent experiments.

Table 4 Effect of Fe^{3+} ion- compared to Cu^{2+} ion-containing systems on 8oxodG formation by PB-DOPA

$CuSO_4$ and $FeCl_3$ were incubated with 200 μg calf-thymus DNA in 1 ml solution with the following final reactants concentration: DNA, DNA + metal ion (500 μM); + DOPA, DNA + metal ion (500 μM) + DOPA (10 μM); + Ins-DOPA, DNA + metal ion (500 μM) + Ins-DOPA (5 μM DOPA, 100 μM insulin); + BSA-DOPA, DNA + metal ion (100 μM) + BSA-DOPA (0.5 μM DOPA, 20 μM BSA) as described in the Materials and methods section. The results are from a single experiment, which was representative of two experiments.

	8oxodG/10^5dG $CuSO_4$	8oxodG/10^5dG $FeCl_3$
DNA	24.8	19.1
+ DOPA	454.1	117.0
+ Ins-DOPA	213.0	66.8
+ BSA-DOPA	86.3	26.9

Formation of 8oxodG in DNA by γ-radiolysed insulin solution in the presence of Cu(II): lack of impact of protein-bound hydroperoxides

γ-Radiolysis of insulin in the presence of O_2 produces both DOPA and hydroperoxides; the latter may be present both on the side-chains of certain amino acids (e.g. Val, Leu, Ile, Lys, Glu, Pro, which have shown to be particularly susceptible to peroxidation [5,8,9,32]) or on the backbone at α-carbon sites [33]. Such protein hydroperoxides, exposed to redox-active metal ions, have been shown to produce free radicals [34–36] and it was therefore of interest to investigate if the presence of these additional reactive species modified the yield of oxidised DNA damage compared with tyrosinase-treated insulin, which lacks the hydroperoxide groups.

As shown in Table 5, the amount of 8oxodG formed in the presence of either tyrosinase-treated, or γ-irradiated, insulin samples containing comparable amounts of PB-DOPA was similar. Thus the hydroperoxide species present on the γ-irradiated protein do not appear to give rise to a significant enhancement in the yield of 8oxodG formation in DNA in the presence of Cu^{2+} ions. To confirm this observation, the protein hydroperoxides present on γ-irradiated insulin were reduced by $NaBH_4$ to the stable hydroxide products; the samples were subsequently applied to a PD-10 column to remove excess $NaBH_4$. PB-DOPA species would thus be the main reactive compounds present in the γ-irradiated and reduced insulin samples. Interestingly, the yield of 8oxodG formed in DNA samples incubated with γ-irradiated and reduced insulin was four-fold higher than with the non-reduced samples. Possible explanations for this finding were, that $NaBH_4$ reduction generated other reactive reducing species on native insulin, such as thiols from disulphide bonds, that the excess $NaBH_4$ was not completely removed from the protein samples by the column treatment and was playing some role in the reactions, or that the

Table 5 8oxodG formation in DNA by γ-radiolysed insulin in the presence of Cu(II) and the influence of reduction

The samples were applied to a PD-10 column, and the protein containing fractions were collected. The reaction with DNA was then performed as described in the Materials and methods section with the following final reactant concentrations: 200 μg calf-thymus DNA, 75 μM insulin, 6 μM DOPA moiety in tyrosinase-treated and γ-irradiated sample, 500 μM $CuSO_4$. Results are means $\pm$ S.D. of at least three independent experiments. R, sample reduced by NaBH; Ins, native insulin; Tyr + $NaBH_4$, tyrosinase-treated insulin after filtration through a PD-10 column added to the corresponding protein fractions obtained after applying an $NaBH_4$ solution to a PD-10 column.

	8oxodG/10^5dG
γ-radiolysed insulin	68.3 $\pm$ 24.4
γ-radiolysed insulin R	249.2 $\pm$ 58.8
Tyrosinase-treated insulin	100.6 $\pm$ 17.0
Tyrosinase-treated insulin R	354.0 $\pm$ 39.7
Tyr + $NaBH_4$	94.0 $\pm$ 4.8
Ins	6.0 $\pm$ 0.6
Ins R	9.9 $\pm$ 1.8

γ-irradiation generated further oxidation products of protein-bound DOPA such as DOPA–quinone and indole–quinone, as postulated previously [4], which are unable to reduce Cu(II) to initiate oxidative damage to DNA unless they are reduced by $NaBH_4$ back into the active catechol form.

To test these hypotheses several different experiments were performed. Native insulin and native, reduced insulin were incubated with Cu(II) and DNA. No significant increase was observed with the reduced protein, hence the possible role played by reducing species other than DOPA could be discounted. A $NaBH_4$ solution in water was applied to a PD-10 column, and the fraction corresponding to the protein was collected and added to the tyrosinase-treated insulin solution. After incubation with DNA and Cu(II) the amount of 8oxodG was measured and was the same as that with tyrosinase-treated insulin alone. Thus the protein fraction from the PD-10 column was not contaminated by agents capable of inducing DNA oxidation. To test the idea that a DOPA-containing protein might give rise to further DOPA upon reduction, tyrosinase-treated insulin was reduced with $NaBH_4$ and applied to a PD-10 column. Incubation of the reduced-insulin solution with DNA and Cu(II) resulted in greatly enhanced yields of 8oxodG compared with the non-reduced sample. This is consistent with the proposal that both γ-irradiation and tyrosinase-treatment generate further oxidation products of the DOPA moieties on protein molecules, and some of these oxidation products are reactivated on treatment with $NaBH_4$. In agreement with this, tyrosinase has been shown to catalyse the hydroxylation of tyrosine residues and the subsequent oxidation to the DOPA–quinone form [13,37]. These results, and also the lack of activity of tyrosinase-treated melittin (a tyrosine-free protein) clearly support the involvement of PB-DOPA species in DNA oxidation.

DISCUSSION

Although a number of isolated studies have previously reported that oxidised proteins can induce damage to others biomolecules ([38–41]; reviewed in [14]), to our knowledge, none has shown that oxidized proteins are able to transfer damage to DNA. Recent studies from our laboratories have identified two reactive species on proteins, protein hydroperoxides and protein bound-DOPA, which may play a significant role in promoting radical induced reactions on other cellular targets. Among all the oxidized amino acids so far identified in the literature, DOPA together with protein carbonyls are the most abundant components in tissue proteins from humans under both physiological and pathological conditions [12,14]. DOPA is also known to serve as a metabolite in the synthesis of melanin [42] and dopamine, one of the neurotransmitters in the central nervous system and which is a precursor of hormones, is formed by decarboxylation of DOPA.

This study provides strong evidence that protein-bound DOPA can be directly involved in oxidative DNA damage *in vitro*. In this way, protein damage by radicals can be linked to mutagenic damage to DNA. Cu ions have been shown to induce significantly more DNA-base damage in the presence of DOPA than do Fe ions, and appear to be a biologically-relevant catalyst for the generation of DNA-damaging species, despite the lower concentration of Cu in the body (typically < 100 mg) than Fe (4 g) [43,44]. Thus Cu is known to be an important structural metal ion in chromatin (and hence in close proximity to DNA), and is present at a concentration of about one ion/kb [45–47]. While virtually all Cu ions in human plasma are bound to caeruloplasmin [48], several functional disorders, such Wilson's disease, can result in the presence of high concentrations of Cu ions in plasma.

It is well established that 8oxodG and 5OHdC formation in DNA can be induced by metal-ion catalysed oxidation systems and by hydroxy radicals generated by γ-irradiation [26,49–51]. We suspect that the mechanism by which DOPA (free or protein-bound) and Cu(II) mediate oxidation of DNA is O_2-dependent and that hydroxy radicals are the DNA-damaging species, and these species are generated through a pseudo-Fenton reaction between H_2O_2 and Cu(I) (reaction 1). The Cu(I) for this reaction is likely to arise from the reduction of Cu(II) by the catecholic (free or protein-bound) DOPA (reaction 2). The occurrence of the latter process was examined by EPR studies on the loss of the (EPR-active) Cu(II) signal in the presence of DOPA (Figure 5); the Cu(I) which arises as a result of reduction is EPR-silent. Incubation of the aquo–Cu(II) complex with DOPA resulted in changes to the Cu(II) signal, which have been ascribed to the formation of a Cu(II)–DOPA complex. No loss of signal intensity (ie. Cu(II) reduction) occurred, however, unless the Cu(I) chelator, neocuproine, was added to the solution [52]; in the presence of this chelator rapid reduction was observed. Cu(II) in the absence of DOPA was not reduced by neocuproine. This result suggests that the catecholic DOPA moieties in proteins play a triggering role in DNA oxidation by driving the reduction of Cu(II) to Cu(I) (i.e. reaction 2). In agreement with this, 3-(2,4-dihydroxyphenyl)propionic acid, a DOPA analogue which is not a catechol, was unable to induce DNA damage in our systems (Table 2). The requirement of a catechol structure in this reaction was suggested previously by investigations with quercetin [53].

The nature of the ligands to which the copper is bound obviously determine the rate and extent of this reduction and as it is well known that Cu ions can bind to DNA [54]. We investigated whether such binding allowed rapid reduction of Cu(II) to Cu(I). EPR studies of Cu(II) reduction by DOPA in the presence of DNA confirmed that this reaction does occur, even in the absence of neocuproine. It has also been also reported that DOPA is able to bind to DNA [16], thus it is possible that a ternary complex of PB-DOPA, Cu(II) and DNA is formed, which generates radicals *in situ* via Cu(I), as shown when quercetin was used as the reducing agent [53,55]. The formation of H_2O_2, which is required for the occurrence of reaction (1), may arise via two possible routes: either as a result of autoxidation of some of the Cu(I) formed via reaction (2) in the presence of molecular O_2 (reaction 3), or via reduction of molecular O_2 by

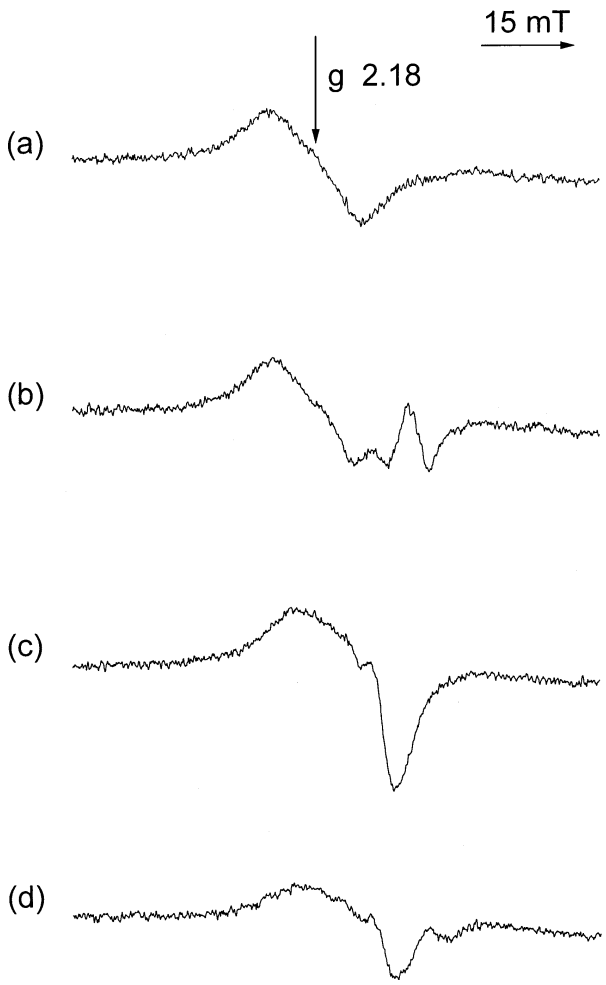

Figure 5 EPR spectra of Cu(II) reduction by DOPA

EPR spectra of Cu(II) ions (666 μM) in the presence or absence of DOPA (333 μM) and neocuproine (2.7 mM). (**a**) Cu(II) ions in water; signal assigned to an aquo–Cu(II) complex. (**b**) Cu(II) ions in the presence of DOPA; signal assigned to a Cu(II)–DOPA complex. (**c**) Cu(II) ions in the presence of neocuproine; signal assigned to Cu(II)–neocuproine complex. (**d**) Cu(II) ions in the presence of DOPA and neocuproine, signal assigned to Cu(II)–neocuproine complex.

the semiquinone from DOPA arising from reaction (2) (reaction 4). The occurrence of either of these reactions (which have been given precedence in the literature [56–59]) would give superoxide radicals and hence hydrogen peroxide via dismutation.

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^- + HO^\bullet \quad (1)$$

$$Cu(II) + DOPA \rightarrow Cu(I) + DOPA^{\bullet -} \quad (2)$$

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\bullet -} \quad (3)$$

$$DOPA^{\bullet -} + O_2 \rightarrow DOPA + O_2^{\bullet -} \quad (4)$$

In vivo, PB-DOPA may be able to undergo repeated catechol–quinone–catechol redox cycles, as has been shown *in vitro* under extreme conditions (glycine as reductant at pH 10) [15]. Biological reductants such as ascorbate, cysteine or glutathione might be expected also to participate in the reduction of the quinone, and hence result in an increased extent of redox cycling and amplification of the radical-generating events. Of course, the relative proximity of protein and DNA is critical for such reactions and the proteins closely apposed to DNA, such as histones and the nuclear-matrix proteins (a proposed major site of Cu binding [47,60]), are the most likely participants. Whether such pro-oxidant reactions can occur *in vivo* may depend on the availability and binding of these metal ions at appropriate reaction sites.

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