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Intense oxidative DNA damage promoted by L-DOPA and its metabolites Implications for neurodegenerative disease

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Abstract Oxidative DNA damage can cause mutation and cell death. We show that L-DOPA, dopamine and 3-*O*-methyl-DOPA cause extensive oxidative DNA damage in the presence of H_2O_2 and traces of copper ions. 8-Hydroxyguanine is the major product. Iron ions were much less effective and manganese ions did not catalyse DNA damage. We propose that copper ion release, in the presence of L-DOPA and its metabolites, may be an important mechanism of neurotoxicity, e.g. in Parkinson's disease and amyotrophic lateral sclerosis.

Key words: DNA damage; Copper; L-DOPA; Hydroxyl radical; Parkinson's disease

1. Introduction

Increased levels of oxidative DNA damage have been reported in Parkinson's disease and Alzheimer's disease and have been suggested to be important in their pathology [1,2]. However, the mechanism of this damage has not been elucidated. Similarly, the familial dominant form of the progressive neurological disease amyotrophic lateral sclerosis (ALS) may involve oxidative damage, in that it is associated with mutations in the gene on chromosome 21 that encodes the copper, zinc-containing isoenzyme of superoxide dismutase [3]. Recent experiments with transgenic animals suggest that the ALS syndrome is not due to lack of SOD activity, but to some toxic property of the mutant Cu,ZnSOD enzymes [4]. It may be that the mutant enzyme binds copper ions less tightly than usual, so that it is released from the enzymes [5]. Copper is neurotoxic, as illustrated by the brain pathology produced in patients with copper overload as a result of Wilson's disease [6].

The toxicity of released copper may involve free radical damage [7,8]. Copper ions promote lipid peroxidation [9,10] and catalyse formation of highly reactive hydroxyl ($OH^{\bullet}$) radicals from hydrogen peroxide (reviewed in [11]). Mixtures of copper ions with H_2O_2 produce DNA damage; strand breaks [12] and chemical changes in the purine and pyrimidine bases, especially conversion of guanine into 8-hydroxyguanine [13].

However, why should increased availability of copper ions be particularly damaging in the nervous system? The nervous system is rich in L-DOPA and its metabolites, dopamine and 3-*O*-methyl-DOPA. We show here that exposure of DNA to these products in the presence of even traces of copper ions causes massive DNA base damage, and we propose that this could be a cytotoxic mechanism when traces of copper are liberated in the brain, e.g. as a result of cell injury [7,8] and perhaps in ALS [5].

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Abbreviations: 5-OH uracil, 5,hydroxyuracil; 5-OH, Me Uracil, 5-(hydroxymethyl)uracil: FapyAdenine, 4,6-diamino-5-formamido pyrimidine; OH Ade, 8-hydroxyadenine; FapyGuanine, 2,6-diamino-4-hydroxy-5 formamidopyrimidine; 8-OH guanine, 8-hydroxyguanine.

2. Materials and methods

2.1. Materials

L-DOPA, dopamine, 3-*O*-methyl-DOPA, 1,10 *o*-phenanthroline, bleomycin sulphate, calf thymus DNA, 8-azaadenine, 6-azathymine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid),4,6-diamino-5-formamidopyrimidine (FAPy-adenine), 2,5,6-triamino-4-hydroxypyrimidine and 5-(hydroxymethyl)uracil were purchased from Sigma Chemical Co (Poole, Dorset, UK). 2-Amino-6,8-dihydroxypurine (8-hydroxyguanine) was from Aldrich (Gillingham, Dorset, UK). Silylation grade acetonitrile and bis(trimethylsilyl) trifluoracetamide (BSTFA) (containing 1% trimethylchlorosilane) were obtained from Pierce Chemical Co (Rockford, IL, USA). 6-Amino-8-hydroxypurine (8-hydroxyadenine) was synthesised by treatment of 8-bromoadenine with concentrated formic acid (95%) at 150°C for 45 min and purified by crystallisation from water. 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FAPy- guanine) was synthesised by treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid and recrystallised from water. Dialysis membranes with a molecular mass cut off of 3500 were purchased from Spectrum supplied by Pierce Chemical Co. Distilled water passed through a purification system (Elga, High Wycombe, Bucks, UK) was used for all purposes.

2.2. Treatment of DNA

The amount of DNA recovered for each sample after dialysis was determined spectrophotometrically at 260 nm (A_{260} of 1.0 = 50 μg DNA/ml). One nmol of 6-azathymine and 2 nmol of 8-azaadenine were added as internal standards to each aliquot containing 0.1 mg of DNA. Aliquots were then lyophilized.

2.3. DNA hydrolysis

DNA aliquots were hydrolysed with 0.5 ml formic acid (60% v/v) in evacuated and sealed tubes for 45 min at 150°C. Samples were lyophilised and then trimethylsilyated in poly(tetrafluorethylene)-capped hypovials (Pierce) with 0.1 ml of a BSTFA/acetonitrile (4:1, v/v) mixture by heating at 90°C for 60 min.

2.4. Gas chromatographylmass spectrometry

Derivatized samples were analysed by a Hewlett-Packard 5971A mass selective detector interfaced with a Hewlett-Packard 5890II gas chromatograph equipped with an automatic sampler and a computer work station. The injection port and the GC-MS interface were kept at 220°C and 280°C, respectively. Separations were carried out on a fused silica capillary column (12 m × 0.20 mm i.d) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33 μm) (Hewlett-Packard). Helium was the carrier gas with a constant flow rate of 0.91 ml/min. Derivatized samples (4 μl) were injected into the GC injection port using the split mode. A split ratio of 8:1 was used, resulting in

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approximately 0.5 μg of DNA loaded on to the column. The column temperature was increased from 145 to 190°C at 10°C/min after 2 min at 145°C. Subsequently the temperature was further increased from 190°C to 270°C at 30°C/min and then kept at 270°C for 2 min. Selected-ion monitoring was performed using the electron-ionisation mode at 70 eV with the ion source maintained at 180°C.

2.5. Assay of metal ions

Brain tissue from control subjects who died from non-neurological disorders and from patients who died from clinically diagnosed Parkinson's disease was obtained from the Parkinson's Disease Society Brain Bank (University Department of Clinical Neurology, Institute of Neurology, London).

At autopsy, brains were removed and divided midsagittally. One half of the brain was immediately frozen at −70°C for biochemical analysis. The substantia nigra, putamen and cerebellum were then dissected on a refrigerated platform (−11°C) from the frozen brain using standard dissection techniques The dissected brain tissue was stored at −70°C until analysis. Samples (20–30 mg) from these parts of the brain were weighed into Eppendorf tubes. One ml of double distilled deionized water was added and the tissue was dispersed by pulse sonication. Control samples consisting of 1 ml of water only were subjected to the same treatment. The homogenates were then assayed for iron and copper ions catalytic for free radical reactions using the bleomycin and copper-phenanthroline assays respectively as described in [14]. Homogenised brain samples peroxidise readily (reviewed in [15]) and create high blanks in the phenanthroline assay. In order to correct for this, butylated hydroxytoluene (0.02% w/v, final concentration) was included in all copper-phenanthroline assays. Calibration experiments were performed in the presence of BHT, which had little effect on the assay.

3. Results

3.1. Oxidative DNA damage; effect of L-DOPA and its metabolites

Oxidative DNA base damage was measured using gas chromatography/mass spectrometry [16], which allows determination of multiple products of free radical attack on the DNA bases [16–18]. Exposure of DNA to L-DOPA, dopamine or 3-*O*-methyl-DOPA did not increase oxidative base damage over that present in the commercial DNA itself (Table 1). Similarly, exposure of the DNA to copper ions or to H_2O_2 alone had little effect (Table 2). As expected [13], Cu^{2+}/H_2O_2 caused some DNA base damage, which was greatly accelerated in the presence of ascorbic acid. However, when DOPA, dopamine or 3-*O*-methyl-DOPA were incubated with DNA, Cu^{2+} and H_2O_2, there was devastating DNA base damage, to an extent we have never observed in our previous work [17,18] (Table 2, last 3 columns). By contrast, mixtures of iron ions with H_2O_2 and dopamine, L-DOPA or 3-*O*-methyl-DOPA produced much less oxidative DNA base damage (Table 3).

Table 1
Lack of effect of L-DOPA, dopamine or 3-*O*-methyl-DOPA on oxidative damage to the bases of DNA

Base product (nmol/mg DNA)	DNA only	DNA + DOPA-MINE	DNA + L-DOPA	DNA + Me DOPA
5-OH Uracil	0.036	0.035	0.045	0.036
5-OH, Me Uracil	0.013	0.023	0.014	0.016
FAPy Adenine	0.190	0.188	0.224	0.339
8-OH Adenine	0.130	0.055	0.106	0.140
FAPy Guanine	0.579	0.385	0.591	0.497
8-OH Guanine	0.104	0.050	0.201	0.141
Total damage	1.052	0.736	1.181	1.169

DOPA and its metabolites were present at final concentrations of 1 mM.

Figs. 1 and 2 show the effect of Cu^{2+} concentration on oxidative DNA damage in the presence of dopamine, L-DOPA or 3-*O*-methyl-DOPA. Significant increases were seen at copper ion concentrations as low as 1 μM. In all cases, the major base damage product was 8-hydroxyguanine (Table 2 and Fig. 2).

Manganese ions are known to promote oxidation of catecholamines [19]. However, replacing copper ions by Mn^{2+} ions in any of the above systems did not significantly increase DNA damage (Table 4).

3.2. Copper and iron availability in brain

Brain tissue was homogenised and the availability of iron and copper ions catalytic for free radical reactions was measured as described in section 2. Table 5 shows that significant levels of both metal ions could be measured, in all 3 brain regions tested, from both parkinsonian brain and control brains matched for sex, age at time of death, storage time and other parameters of tissue handling.

4. Discussion

We show here that copper ions are very powerful inducers of oxidative damage to DNA in the presence of H_2O_2 and DOPA, dopamine or 3-*O*-methyl-DOPA. Hydrogen peroxide is a normal metabolite in brain, produced by several enzymes, including SOD and monoamine oxidase [3,20]. Our data using the phenanthroline assay (Table 5) show that copper ion concentrations in injured brain, although low, are at the level that could promote such damage and small increases in copper ions availability could produce large increases in damage (Figs. 1 and 2). Thus if copper ions were released from mutant Cu,ZnSOD enzymes in ALS, then damage could increase

Table 2
Damage to DNA bases by copper ion/H_2O_2 systems in the presence of ascorbate, dopamine, L-DOPA or 3-*O*-methyl-DOPA

Base product (nmol/mg)	DNA Only	DNA Cu^{2+}	DNA + Cu^{2+} plus				DNA H_2O_2	DNA + Cu^{2+}, H_2O_2	DNA + Cu^{2+}, H_2O_2 plus			
			ASC	Dopamine	L-DOPA	MeDOPA			ASC	Dopamine	L-DOPA	MeDOPA
5-OH Uracil	0.035	0.137	0.349	0.241	0.261	0.267	0.254	0.284	3.544	17.943	21.631	19.441
5-OH, Me Uracil	0.010	0.025	0.046	0.101	0.141	0.099	0.015	0.039	0.348	0.741	0.911	0.732
FAPy Adenine	0.146	0.254	0.518	0.291	0.582	0.563	0.178	0.471	2.082	7.811	11.414	8.914
8-OH Adenine	0.095	0.221	0.517	1.431	1.694	1.563	0.198	0.604	8.076	28.141	31.914	30.631
FAPy Guanine	0.682	0.589	2.319	1.561	1.714	1.704	0.561	0.659	6.972	24.816	22.764	20.119
8-OH Guanine	0.177	0.100	1.554	2.714	3.119	3.431	0.198	0.531	55.640	283.141	362.671	351.617
Total damage	1.145	1.326	5.303	6.339	7.511	7.627	1.404	2.588	76.662	362.593	451.305	431.454

Reaction mixtures contained buffer, calf-thymus DNA, H_2O_2 (2.8 mM) $CuSO_4$ (100 μM) and other products at the final concentrations indicated: ascorbate (100 μM), L-DOPA (100 μM), Dopamine (100 μM), 3-*O*-Methyl-DOPA (100 μM).

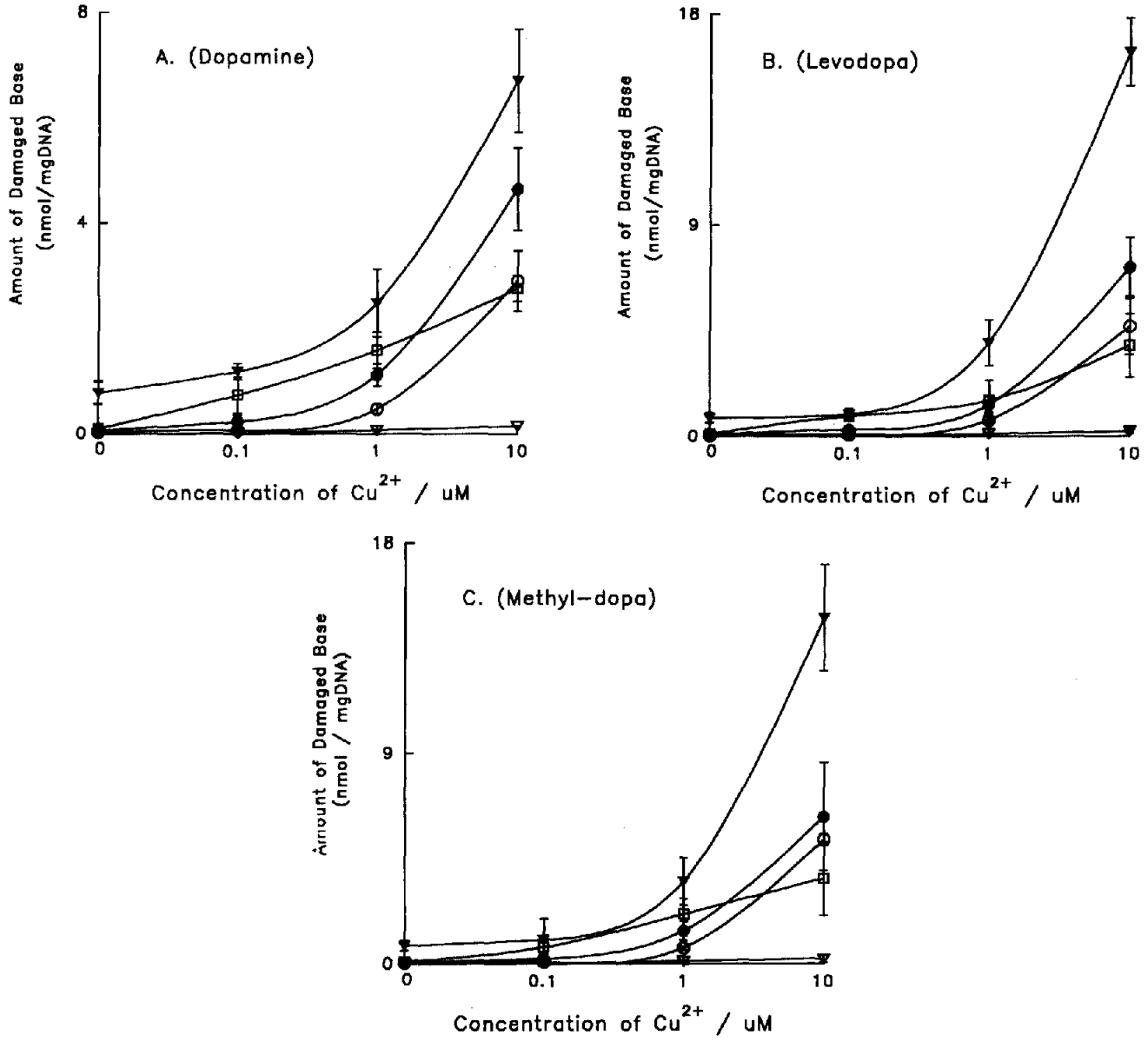

Fig. 1. Effect of increasing copper ion concentrations on DNA base damage in the presence of H_2O_2 and L-DOPA, dopamine or 3-*O*-methyl-DOPA (all at 1 mM final concentration). $CuSO_4$ was present at the final concentrations indicated. FaPy guanine ▼; OH adenine ●; OH uracil ○; FAPy adenine □; OH, Me uracil ▽. A, dopamine; B, L-DOPA; C, 3-*O*-methyl-DOPA.

markedly. Levels of L-DOPA and its metabolites in brain are sufficient to allow the reactions we have described to occur [20,21]. There appeared to be no significant difference in copper ion levels between normal and parkinsonian brain, although the variability in results makes this conclusion tentative because of the small number of samples we have used.

Table 3
Damage to DNA bases by iron ion-containing systems

Base product (nmol/mg)	DNA Only	DNA Fe^{3+}	DNA + Fe^{3+} plus					DNA + Fe^{3+}, H_2O_2 plus			
			ASC	Dopamine	L-DOPA	MeDOPA	H_2O_2	ASC	Dopamine	L-DOPA	MeDOPA
5-OH Uracil	0.036	0.149	0.281	0.151	0.167	0.190	1.015	1.688	0.381	0.404	0.356
5-OH,Me Uracil	0.013	0.070	0.094	0.096	0.136	0.104	0.077	0.147	0.142	0.143	0.116
FAPy Adenine	0.190	1.133	1.445	1.431	1.697	1.506	4.836	4.910	2.291	2.416	2.396
8-OH Adenine	0.130	0.311	0.436	0.468	0.471	0.490	1.011	1.325	0.941	1.316	1.104
FAPy Guanine	0.579	0.776	0.871	0.986	1.631	1.143	0.841	7.514	4.041	10.461	5.914
8-OH Guanine	0.104	2.040	3.773	1.843	1.948	2.046	5.738	20.466	15.941	16.114	16.748
Total damage	1.052	4.479	6.900	4.975	6.050	5.479	13.518	36.050	23.737	30.854	26.634

Experiments were carried out as described in the legend to Table 2, except that $CuSO_4$ was replaced by $FeCl_3$.

Table 4
Lack of damage to DNA bases by manganese ion-containing systems

Base product (nmol/mg)	DNA Only	DNA + Mn^{2+}	DNA + Mn^{2+} plus					DNA + Mn^{2+}, H_2O_2 plus			
			ASC	Dopamine	L-DOPA	MeDOPA	H_2O_2	ASC	Dopamine	L-DOPA	MeDOPA
5-OH Uracil	0.031	0.040	0.034	0.024	0.027	0.103	0.069	0.073	0.070	0.094	0.071
5-OH,Me Uracil	0.014	0.012	0.021	0.011	0.018	0.024	0.021	0.017	0.018	0.034	0.016
FAPy Adenine	0.231	0.247	0.381	0.464	0.394	0.463	0.333	0.479	0.474	0.391	0.446
8-OH Adenine	0.111	0.250	0.112	0.060	0.169	0.261	0.233	0.286	0.221	0.351	0.247
FAPy Guanine	0.715	1.253	0.936	1.016	0.970	1.827	1.941	2.155	1.920	2.043	2.001
8-OH Guanine	0.205	0.649	0.324	0.230	0.271	0.331	0.874	0.564	0.499	1.149	0.508
Total damage	1.307	2.451	1.808	1.805	1.849	3.009	3.471	3.574	3.202	4.062	3.289

Experiments were carried out as described in the legend to Table 2, except that $CuSO_4$ was replaced by $MnSo_4$ and the compounds were tested at the final concentration of 1 mM.

The pattern of DNA damage observed is characteristic of attack by highly-reactive hydroxyl (OH•) radicals, giving a multiplicity of base damage products [16–18]. Many of these are known to be mutagenic or to interfere with DNA replication [22,23]. Excessive DNA damage can lead not only to mutations but also to cell death, e.g. by NAD^+ depletion [24]. The major product was 8-hydroxyguanine; this product may be favoured because of binding of Cu^{2+} ions to GC-rich regions in DNA [25]. Indeed, the binding of copper ions to DNA may facilitate their interaction with L-DOPA and its metabolites in a way that greatly accelerates free radical production [26].

Iron ions also promoted oxidative DNA damage in the presence of L-DOPA and its metabolites, although to a much lesser extent than Cu^{2+} (Table 3). However, our data with the bleomycin assay show that comparable amounts of iron and copper ions are released from injured human brain tissue. Gutteridge et al. [27] reported that 19–25 nmol/g of bleomycin-detectable iron is present in gerbil brain tissue, results comparable to our human brain samples (Table 5). It seems, therefore, that iron-dependent reactions might be of lesser significance than copper-dependent reactions in vivo.

Although manganese ions are known to promote catecholamine oxidation, no oxidative DNA base damage was observed in this system. This may be because Mn^{2+} ions will not convert H_2O_2 to OH• [28]. Our data may explain the elevated DNA damage seen in several neurodegenerative diseases, and could be particularly relevant to Parkinson's disease. Treatment of this disease with L-DOPA will raise levels of L-DOPA, dopamine and 3-*O*-methyl-DOPA in the brain [20,21]. Thus recent reports that levels of 8-hydroxyguanine are increased in DNA from parkinsonian substantia nigra [21] do not necessarily mean that the disease itself increases oxidative DNA damage, but could be due (in whole or in part) to the treatment.

Table 5
Measurement of iron and copper ions catalytic for free radical reactions in human brain tissue

Brain Region	Iron detected in bleomycin assay		Copper detected in phenanthroline assay	
	μM in assay	nmol/g tissue	μM in assay	nmol/g tissue
Cerebellum				
normal	0.72 ± 0.02	26 ± 5 (6)	0.60 ± 0.35	21 ± 10 (6)
PD	0.71 ± 0.16	25 ± 7 (6)	0.83 ± 0.21	29 ± 7 (6)
Median putamen				
normal	1.86 ± 0.57	61 ± 20 (6)	0.45 ± 0.37	21 ± 16 (6)
PD	1.19 ± 0.54	45 ± 17 (6)	1.53 ± 0.93	56 ± 30 (6)
Substantia nigra				
normal	1.27 ± 0.73	51 ± 27 (3)	1.20 ± 0.80	53 ± 48 (3)
PD	0.59 ± 0.07	36 ± 23 (3)	1.15 ± 1.15	63 ± 48 (2)

A known mass of brain tissue (20–30 mg) was dissected from the regions shown above and sonicated in 1 ml of double distilled water. Copper and iron determinations were then carried out immediately on the homogenate as described in section 2. Controls consisting of 1 ml of distilled water taken through the same procedure had no detectable iron or copper. Results are mean ± S.D., *n* in parentheses. PD = Parkinson's disease.

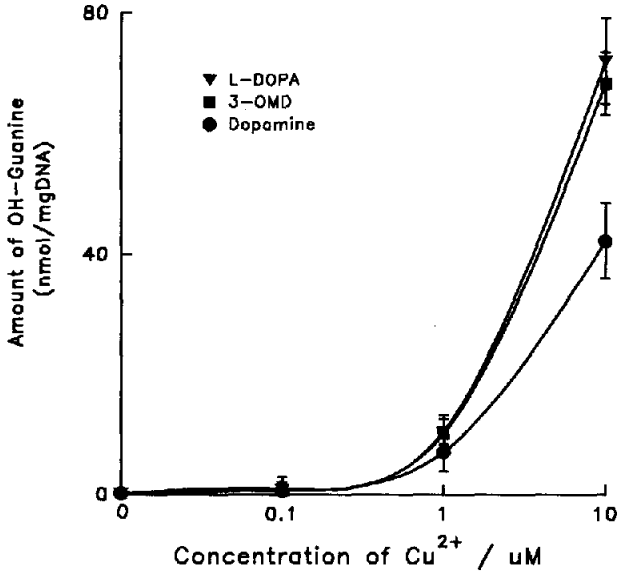

Fig. 2. Effect of increasing copper ion concentrations on 8-hydroxyguanine concentrations in DNA in the presence of H_2O_2 and L-DOPA, dopamine or 3-*O*-methyl-DOPA (all at 1 mM final concentrations).

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