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Mechanism of metal-mediated DNA damage and apoptosis induced by 6-hydroxydopamine in neuroblastoma SH-SY5Y cells

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Abstract

6-Hydroxydopamine (6-OHDA) is a neurotoxin to produce an animal model of Parkinson's disease. 6-OHDA increased the formation of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), a biomarker of oxidatively damaged DNA, and induced apoptosis in human neuroblastoma SH-SY5Y cells. Iron or copper chelators inhibited 6-OHDA-induced 8-oxodG formation and apoptosis. Thus, iron and copper are involved in the intracellular oxidatively generated damage to DNA, a stimulus for initiating apoptosis. This study examined DNA damage caused by 6-OHDA plus metal ions using ^{32}P-5′-end-labelled DNA fragments. 6-OHDA increased levels of oxidatively damaged DNA in the presence of Fe(III)EDTA or Cu(II). Cu(II)-mediated DNA damage was stronger than Fe(III)-mediated DNA damage. The spectrophotometric detection of *p*-quinone and the scopoletin method showed that Cu(II) more effectively accelerated the 6-OHDA auto-oxidation and H_2O_2 generation than Fe(III)EDTA. This study suggests that copper, as well as iron, may play an important role in 6-OHDA-induced neuronal cell death.

Keywords: *6-hydroxydopamine, Parkinson's disease, apoptosis, DNA damage, copper, iron*

Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra. The dopamine analogue 6-hydroxydopamine (6-OHDA) is one of the commonly used neurotoxins to produce animal models of PD. In rodents and primates, many parkinsonian-like neurological features can be produced by selective toxicity of 6-OHDA toward dopaminergic neuronal cells [1–3]. It is well known that iron plays an important role in 6-OHDA cytotoxicity [4]. In rats, iron chelators protected against lesions of striatal dopaminergic neurons induced by 6-OHDA [5]. Derivatives of neuropeptide with iron chelating ability protected PC12 cells against 6-OHDA cytotoxicity [6]. Furthermore, some reports indicate that copper may participate in neuronal cell death induced by 6-OHDA. The loss of dopaminergic neurons induced by 6-OHDA was aggravated in metallothionein knock-out mice [7]. The metallothionein family is believed to be implicated in metabolism of heavy metals such as copper and zinc in most tissue, including brain [8,9]. These studies suggest that metals are involved in dopaminergic neurodegeneration caused by 6-OHDA.

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Oxidatively damaged DNA is a well-known stimulus for initiating apoptosis in neuron [10,11]. 6-OHDA increased oxidative DNA base modifications in the brain [12,13], urine [13] and serum [13] of rats. Treatment of 6-OHDA led to DNA strand breaks formation in neuroblastoma cell line SK-N-SH [14]. Recently, it was revealed that dopamine [15] and its metabolite tetrahydropapaveroline [16] induced DNA damage and apoptosis in the presence of metal ions. Therefore, it is considered that oxidatively generated damage to DNA mediated by metals may be implicated in the mechanism of 6-OHDA-induced dopaminergic neuronal cell death. However, the mechanism of metal-mediated DNA damage induced by 6-OHDA is still not fully understood.

In this study, to clarify the involvement of metal ions in oxidatively generated damage to DNA and apoptosis induced by 6-OHDA, we analysed the effect of metal chelators on 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) formation and DNA ladder formation in 6-OHDA-treated SH-SY5Y cells, a human neuroblastoma cell line. In addition, to elucidate the mechanism of intracellular oxidatively generated damage to DNA, we examined 6-OHDA-induced DNA damage and its site specificity in the presence of metal ions using ^{32}P-5′-end-labelled DNA fragments. We also investigated the 6-OHDA auto-oxidation and H_2O_2 generation in the presence of metal ions by the spectrophotometric method and the scopoletin method [17], respectively.

Materials and methods

Materials

Restriction enzymes and calf intestine phosphatase (CIP) were purchased from Boehringer Mannheim GmbH (Germany). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). [γ-^{32}P] ATP was from New England Nuclear (Boston, MA). 6-OHDA was from Aldrich Chemical Co. (Milwaukee, WI). Diethylenetriamine-*N,N,N′,N″, N‴*-pentaacetic acid (DTPA) and bathocuproinedisulphonic acid were from Dojin Chemical Co. (Kumamoto, Japan). Acrylamide, dimethylsulphoxide (DMSO), bisacrylamide, nuclease P_1 and piperidine were from Wako Pure Chemical Industries (Osaka, Japan). Proteinase K was from Merck (Darmstadt, Germany). $CuCl_2$, Fe(III)EDTA, scopoletin, ethanol, D-mannitol and sodium formate were from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, superoxide dismutase (SOD), bacterial alkaline phosphatase (BAP, from Escherichia coli), RNase A, horseradish peroxidase, D-penicillamine and catalase were from Sigma Chemical Co. (St. Louis, MO). 3-Methylthiopropionaldehyde (methional) was from Tokyo Kasei (Tokyo, Japan). Deferoxamine mesilate was from Novartis Pharma (Tokyo, Japan).

Measurement of 8-oxodG in cultured cells treated with 6-OHDA

The human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). SH-SY5Y cells were grown in Minimum Essential Medium: F-12 Nutrient Mixture (1:1) containing 15% foetal bovine serum, 1% non-essential amino acids and 100 mg/l kanamycin at 37°C under 5% CO_2 in a humidified atmosphere. SH-SY5Y cells (2×10^6 cells) were incubated with 6-OHDA at 37°C and, after incubation, immediately washed three times with phosphate-buffered saline (PBS). Where indicated, SH-SY5Y cells were pre-incubated with 250 μM deferoxamine at 37°C for 30 min or with 1 mM penicillamine at 37°C for 12 h. The cells were then suspended in 50 μg/ml RNase, 500 μg/ml proteinase K and 500 μl Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA), followed by incubation at 60°C for 1 h under anaerobic conditions. After ethanol precipitation, DNA was digested to the nucleosides with 8 units nuclease P_1 and 1.2 units BAP in anaerobiosis and analysed using HPLC-ECD by modification of a reported method [18] as described previously [19].

Detection of DNA ladder formation induced by 6-OHDA

SH-SY5Y cells (1×10^6 cells) were incubated with 6-OHDA at 37°C. Where indicated, SH-SY5Y cells were pre-incubated with 250 μM deferoxamine at 37°C for 30 min or with 1 mM penicillamine at 37°C for 12 h. After incubation, the cells were immediately washed three times with PBS and lysed in a solution of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.5% sodium dodecyl sulphate. The lysate was treated with 200 μg/ml RNase at 56°C for 1 h and 250 μg/ml proteinase K at 37°C for 1 h. The DNA was precipitated with 2.5 volumes of ethanol and electrophoresed on a 1.4% agarose gel containing ethidium bromide.

Preparation of ^{32}P-5′-end-labelled DNA fragments

DNA fragments containing exon 2 of the human *p16* tumour suppressor gene [20] were obtained as described previously [21]. The 5′-end-labelled 460-base pair fragment (*Eco*RI* 9481-*Eco*RI* 9940) containing exon 2 was further digested with *Bss*HII to obtain the singly labelled 309-base pair fragment (*Eco*RI* 9481-*Bss*HII 9789) and the 147-base pair fragment (*Bss*HII 9794-*Eco*RI* 9940).

DNA fragments were also obtained from the human *p53* tumour suppressor gene. The ^{32}P-5′-end-labelled 650-base pair (*Hin*dIII* 13972-*Eco*RI*

14621) fragments were obtained as described previously [22]. The 650-base pair fragment was digested with *Apa*I to obtain the singly labelled 211-base pair (*Hin*dIII* 13972-*Apa*I 14182) and 443-base pair (*Apa*I 14179-*Eco*RI* 14621) DNA fragments.

DNA fragments were prepared from the plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene. The singly labelled 261-base pair fragment (*Ava*I* 1645-*Xba*I 1905), 341-base pair fragment (*Xba*I 1906-*Ava*I* 2246), 98-base pair fragment (*Ava*I* 2247-*Pst*I 2344) and 337-base pair fragment (*Pst*I 2345-*Ava*I* 2681) were obtained as previously described [23,24]. For reference, nucleotide numbering starts with the *Bam*HI site [25]. An asterisk indicates ^{32}P-labelling.

Detection of damage to ^{32}P-labelled DNA fragments caused by 6-OHDA in the presence of metal ions

The standard reaction mixtures containing ^{32}P-5′-end-labelled DNA fragments, 20 μM/base of calf thymus DNA, 20 μM metal ion and 6-OHDA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated at 37°C for 1 h. Subsequently, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel as described previously [23,24]. The autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure [26], using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

UV-visible spectra measurement during auto-oxidation of 6-OHDA

UV-visible spectra of 6-OHDA were measured with a UV-visible spectrometer (UV-2500PC, Shimadzu, Kyoto). The reaction mixture contained 500 μM 6-OHDA and 5 μM DTPA. Where indicated, 20 μM metal ion was added to the reaction mixtures. The spectra of the mixtures were measured repeatedly at 37°C for the indicated duration.

Detection of H_2O_2 generated by 6-OHDA in the presence of metal ions

The amounts of H_2O_2 were determined by measuring the extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase [17]. Reactions were performed in cuvettes containing 20 μM scopoletin, 5 μM horseradish peroxidase, 5 μM DTPA and 10 μM 6-OHDA with or without 20 μM metal ion at 37°C for 5 min. Fluorescence was measured with a spectrofluorometer (RF-5300PC; Shimadzu, Kyoto, Japan) with sample excitation at 365 nm and emission at 450 nm. In both methods, calibration curves were obtained using H_2O_2 of known concentrations.

Results

Formation of 8-oxodG in cultured cells by 6-OHDA

Figure 1 shows 8-oxodG formation in SH-SY5Y cells treated with 6-OHDA. The content of 8-oxodG in DNA extracted from SH-SY5Y cells treated with 6-OHDA for 2 h was significantly increased in comparison with no treated cells. The amount of 8-oxodG in the no treated (control) cells was 0.39 ± 0.038 per 10^5 dG, which is reasonable when compared with the value reported by European Standards Committee of Oxidative DNA Damage (ESCODD) [27]. To examine the possible role of iron and copper in 6-OHDA-induced 8-oxodG formation, SH-SY5Y cells were pre-incubated with deferoxamine, an iron chelator, or penicillamine, a relatively specific copper chelator, and subsequently incubated with 6-OHDA for 2 h. The pre-incubation with deferoxamine or penicillamine resulted in the significant decrease in the 8-oxodG formation induced by 6-OHDA.

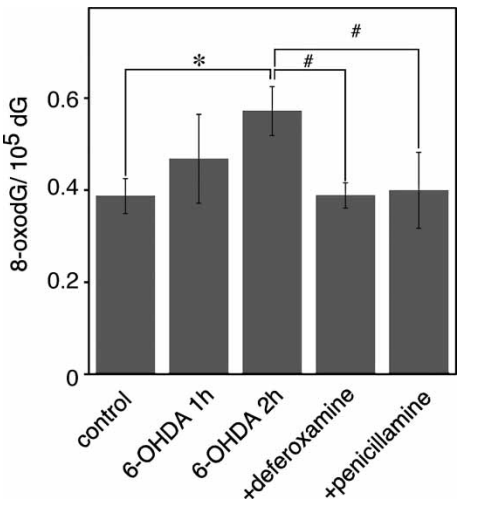

Figure 1. Formation of 8-oxodG in SH-SY5Y cells treated with 6-OHDA. SH-SY5Y cells (2×10^6 cells) were incubated with 50 μM 6-OHDA at 37°C for indicated time. Where indicated, SH-SY5Y cells were pre-incubated with 250 μM deferoxamine at 37°C for 30 min or with 1 mM penicillamine at 37°C for 12 h, followed by incubation with 50 μM 6-OHDA at 37°C for 2 h. The DNA was extracted immediately and digested to nucleosides enzymatically. 8-oxodG content was analysed by HPLC-ECD, as described in Materials and methods. Results are expressed as mean ± SE of values obtained from six independent experiments. Symbols indicate significant differences compared with control ($*p < 0.05$) and significant differences compared with cells treated with 6-OHDA ($\#p < 0.05$) by *t*-test.

DNA ladder formation induced by 6-OHDA

Figure 2 shows the DNA ladder formation, characteristic for apoptosis, induced by 6-OHDA in SH-SY5Y cells. DNA ladder formation was detected at 4 h and 6 h in SH-SY5Y cells treated with 6-OHDA (Figure 2A). Deferoxamine or penicillamine almost completely prevented the DNA ladder formation induced by 6-OHDA (Figure 2B).

Damage to ^{32}P-labelled DNA fragments induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II)

Figure 3 shows the autoradiogram of DNA fragments treated with 6-OHDA in the presence of Fe(III)EDTA or Cu(II). Oligonucleotides were detected on the autoradiogram as a result of DNA damage. The intensity of the DNA damage increased with 6-OHDA concentration in the presence of Fe(III)EDTA or Cu(II). Piperidine treatment increased the amount of oligonucleotides formed. Since altered base is readily removed from its sugar by the piperidine treatment, it is reasonable to conclude that the base modification was induced by 6-OHDA plus Fe(III)EDTA or Cu(II). However, even without treatment, oligonucleotides were formed by 6-OHDA, indicating breakage of the 2-deoxyribose phosphate backbone. The efficiency of both base modification and DNA strand cleavage was higher in the presence of Cu(II) than Fe(III)EDTA. 6-OHDA did not cause DNA damage in the presence of other metal ions (Fe(III), Mn(II), Co(II) or Ni(II)) (data not shown).

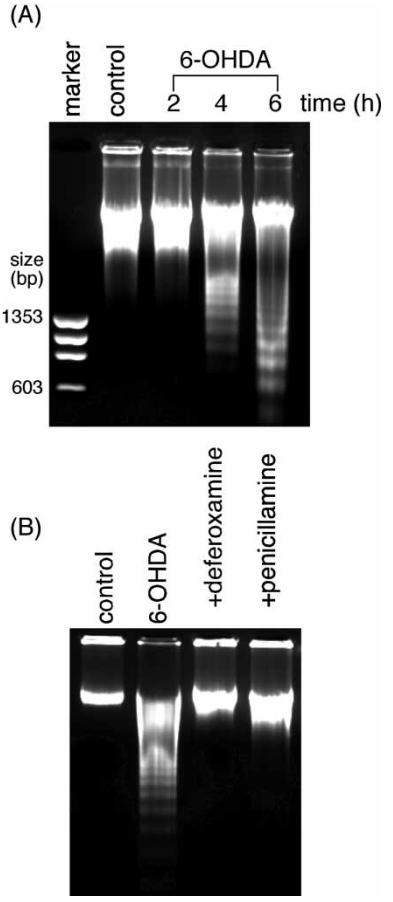

Figure 2. Detection of DNA ladder formation in SH-SY5Y cells treated with 6-OHDA. (A) SH-SY5Y cells (1×10^6 cells) were treated with 50 μM 6-OHDA at 37°C for indicated time. (B) SH-SY5Y cells (1×10^6 cells) were pre-incubated with 250 μM deferoxamine at 37°C for 30 min or with 1 mM penicillamine at 37°C for 12 h, followed by incubation with 50 μM 6-OHDA at 37°C for 6 h. The cells were lysed and DNA was extracted and analysed by conventional electrophoresis as described in Materials and methods. Control: no treated cells. Marker: size marker DNA (ΦX 174/Hae III digest).

Effects of scavengers and metal chelators on DNA damage induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II)

Fe(III)EDTA-mediated DNA damage caused by 6-OHDA was inhibited by •OH scavengers such as ethanol, mannitol, sodium formate, DMSO and methional (Figure 4A). Catalase and deferoxamine also inhibited the DNA damage (Figure 4A). SOD did not inhibit the DNA damage (Figure 4A).

In the presence of Cu(II), typical •OH scavengers (ethanol, mannitol, sodium formate and DMSO) did not prevent the DNA damage by 6-OHDA, while methional inhibited the DNA damage (Figure 4B). DNA damage was inhibited by catalase and bathocuproine, a Cu(I) chelator (Figure 4B). SOD showed little or no inhibitory effect on DNA damage (Figure 4B).

Site specificity of DNA damage induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II)

Figure 5 shows the patterns of DNA cleavage induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II). The relative intensity of DNA damage was obtained by scanning autoradiogram with a laser densitometer. Fe(III)EDTA-mediated DNA damage induced by 6-OHDA occurred at every nucleotide in DNA fragments treated with piperidine (Figure 5A). In contrast, Cu(II)-mediated DNA damage induced by 6-OHDA occurred frequently at thymine and cytosine residues with piperidine treatment (Figure 5B).

Formation of 6-OHDA p*-quinone in the presence of Fe(III)EDTA or Cu(II)*

Figure 6A shows the UV-visible spectral changes of 6-OHDA in the presence or absence of metal ions. It has been reported that the *p*-quinone, which is

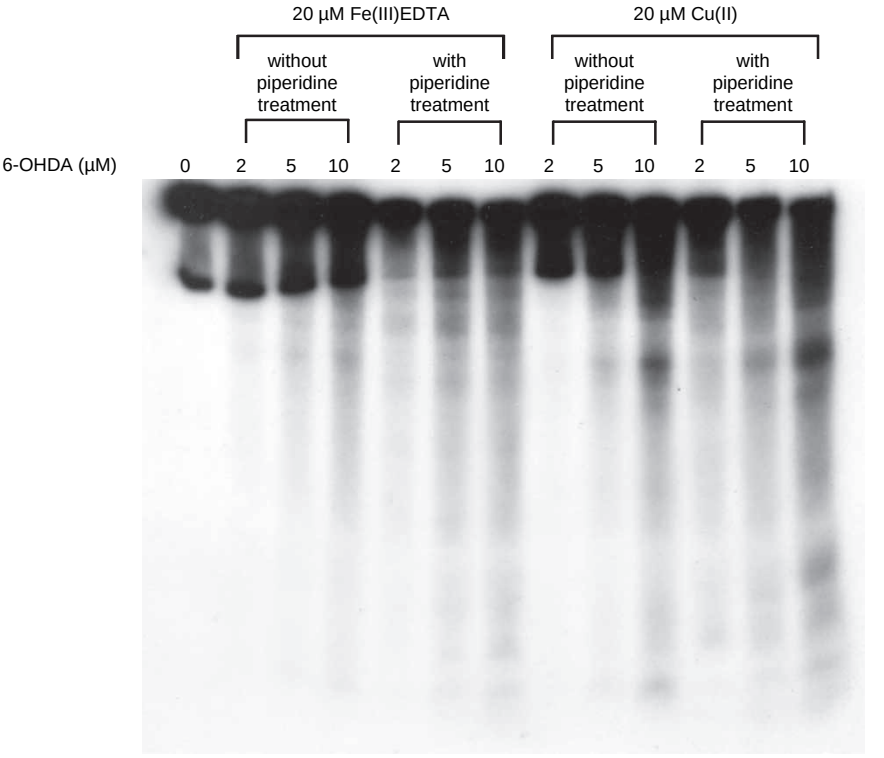

Figure 3. Autoradiogram of ^{32}P-labelled DNA fragments incubated with 6-OHDA in the presence of Fe(III)EDTA or Cu(II). The reaction mixture contained the ^{32}P-labelled 337 base pair fragments, 20 μM/base of calf thymus DNA, the indicated concentrations of 6-OHDA and 20 μM metal ion in 200 μl of 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated at 37°C for 1 h. The DNA fragments were treated with piperidine or without piperidine and electrophoresed on a polyacrylamide gel.

formed through 6-OHDA auto-oxidation, exhibits the characteristic absorption spectrum at ca. 490 nm [28]. In the presence of no metals, 6-OHDA showed a gradual increase in the absorption bands at 490 nm. Addition of Cu(II) or Fe(III)EDTA enhanced the increase of the absorption at 490 nm. The enhancement effect of Cu(II) on the spectra change was higher than that of Fe(III)EDTA.

H_2O_2 generation induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II)

Figure 6B shows H_2O_2 generation caused by 6-OHDA incubated with or without metal ions. In the presence of Cu(II) or Fe(III)EDTA, 6-OHDA-induced H_2O_2 generation was increased in comparison with that in the absence of metal ions. Addition of Cu(II) more effectively enhanced the H_2O_2 generation than Fe(III)EDTA.

Discussion

In this study, we demonstrated that 6-OHDA increased the amount of 8-oxodG in SH-SY5Y cells. 8-oxodG is used as a biomarker of oxidatively damaged DNA [29,30]. An iron chelator deferoxamine significantly prevented elevation of 8-oxodG content by 6-OHDA. A copper chelator penicillamine also significantly inhibited formation of 8-oxodG by 6-OHDA. These results indicated the involvement of iron and copper in 8-oxodG formation in SH-SY5Y cells treated with 6-OHDA. Levay et al. [31] reported that 6-OHDA and Cu(II) caused 8-oxodG formation in isolated DNA. Furthermore, we showed that 6-OHDA treatment resulted in induction of apoptosis in SH-SY5Y cells. 6-OHDA induced-apoptosis was inhibited by the pre-incubation with deferoxamine or penicillamine. This result implied that iron and copper participate in the apoptosis by 6-OHDA. Our group previously demonstrated that oxidatively damaged DNA is associated with apoptosis, through the mitochondrial membrane potential ($\Delta\Psi$m) increase and subsequent caspase-3 activation [32,33]. Therefore, it is reasonably considered that metal-mediated DNA damage is involved in neuronal cell death induced by 6-OHDA.

To clarify the mechanism leading to increase of oxidatively damaged DNA induced by 6-OHDA, we investigated DNA damage by 6-OHDA in the presence of metal ions using ^{32}P-labelled DNA fragments. We demonstrated that 6-OHDA caused DNA damage in the presence of Fe(III)EDTA or Cu(II). In

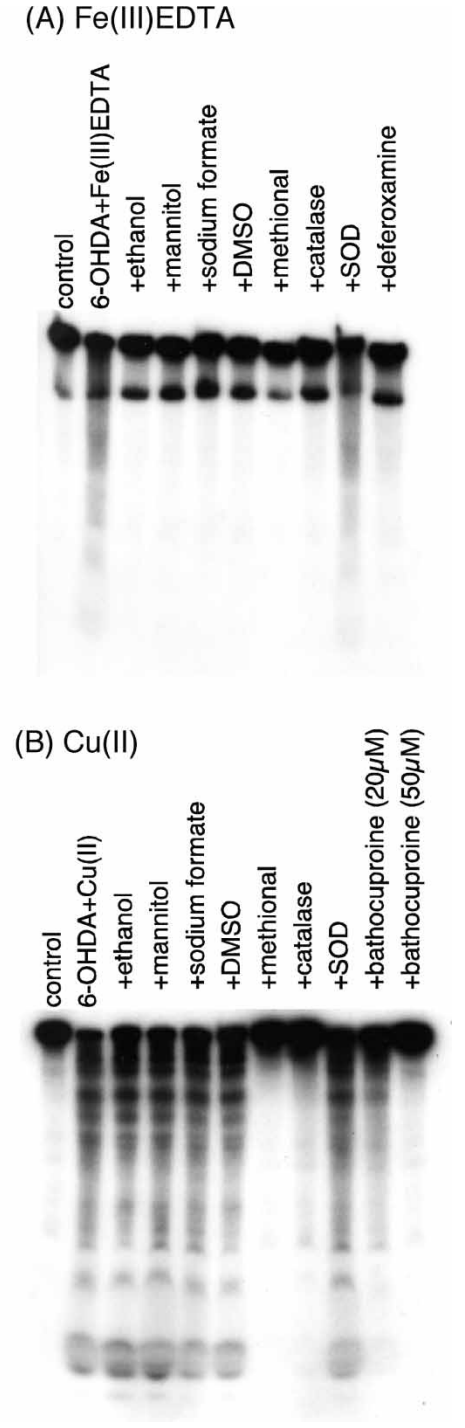

Figure 4. Effect of scavengers and metal chelators on DNA damage induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II). Reaction mixtures contained the ^{32}P-labelled 341 base pair (A) or 443 base pair (B) DNA fragments, 20 μM/base calf thymus DNA, 10 μM 6-OHDA and 20 μM Fe(III)EDTA (A) or $CuCl_2$ (B) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated at 37°C for 1 h. DNA fragments were treated with 1 M piperidine at 90°C for 20 min, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel. The concentrations of scavengers and metal chelators were as follows, 0.8 M ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.8 M DMSO, 0.1 M methional, 30 U catalase, 30 U SOD, 20 or 50 μM bathocuproine, 1 mM deferoxamine.

the presence of Fe(III)EDTA, 6-OHDA induced DNA damage uniformly at every nucleotide. •OH is known to induce sequence-independent DNA cleavage [34–36]. To confirm the participation of •OH in Fe(III)EDTA-mediated DNA damage, we examined the effects of scavengers and an iron chelator on the DNA damage induced by 6-OHDA in the presence of Fe(III)EDTA. Catalase, deferoxamine and •OH scavengers inhibited Fe(III)EDTA-mediated DNA damage, indicating that H_2O_2, iron and •OH are critical for the DNA damage. In contrast, 6-OHDA caused DNA damage at thymine and cytosine in the presence of Cu(II). The DNA damage caused by 6-OHDA plus Cu(II) was inhibited by catalase and bathocuproine. Typical •OH scavengers showed little or no inhibitory effect on the DNA damage induced by 6-OHDA plus Cu(II), suggesting that •OH does not play an important role in Cu(II)-mediated DNA damage. Methional, which scavenges not only •OH but also species with weaker reactivity than •OH [37], inhibited the DNA damage. Therefore, we considered that H_2O_2, Cu(I) and weaker reactive oxygen species than •OH participate in Cu(II)-mediated DNA damage by 6-OHDA. Frelon et al. [38] reported that •OH is not the main reactive species involved in the degradation of DNA bases by copper in the presence of H_2O_2.

Under physiological conditions, 6-OHDA is non-enzymatically oxidized by molecular oxygen to form H_2O_2 and the corresponding *p*-quinone [39]. Recently, it was reported that not only H_2O_2 but also generated secondary products including *p*-quinone are involved in the neuronal cell death caused by 6-OHDA [40]. We showed that the auto-oxidation of 6-OHDA was accelerated in the presence of Fe(III)EDTA or Cu(II) by monitoring the formation of the *p*-quinone. The oxidation rate of 6-OHDA mediated by Cu(II) was much faster than that by Fe(III)EDTA. In addition, the scopoletin method revealed that addition of Cu(II) or Fe(III)EDTA elevated the level of H_2O_2 generated by 6-OHDA. In the presence of Cu(II), the level of H_2O_2 was higher when compared with Fe(III)EDTA. These results are compatible with the observation that the DNA damage caused by 6-OHDA plus Cu(II) was stronger than that by 6-OHDA plus Fe(III)EDTA. Relevantly, we previously reported a similar effect of Cu(II), which showed a stronger activity in endogenous reductants-induced DNA damage than Fe(III)EDTA [41]. These results suggested that copper plays an important role in 6-OHDA cytotoxicity as well as iron.

On the basis of these results, the possible mechanisms of metal-mediated DNA damage induced by 6-OHDA are proposed in Figure 7. Metal ion (M^n) mediates 6-OHDA auto-oxidation, leading to the

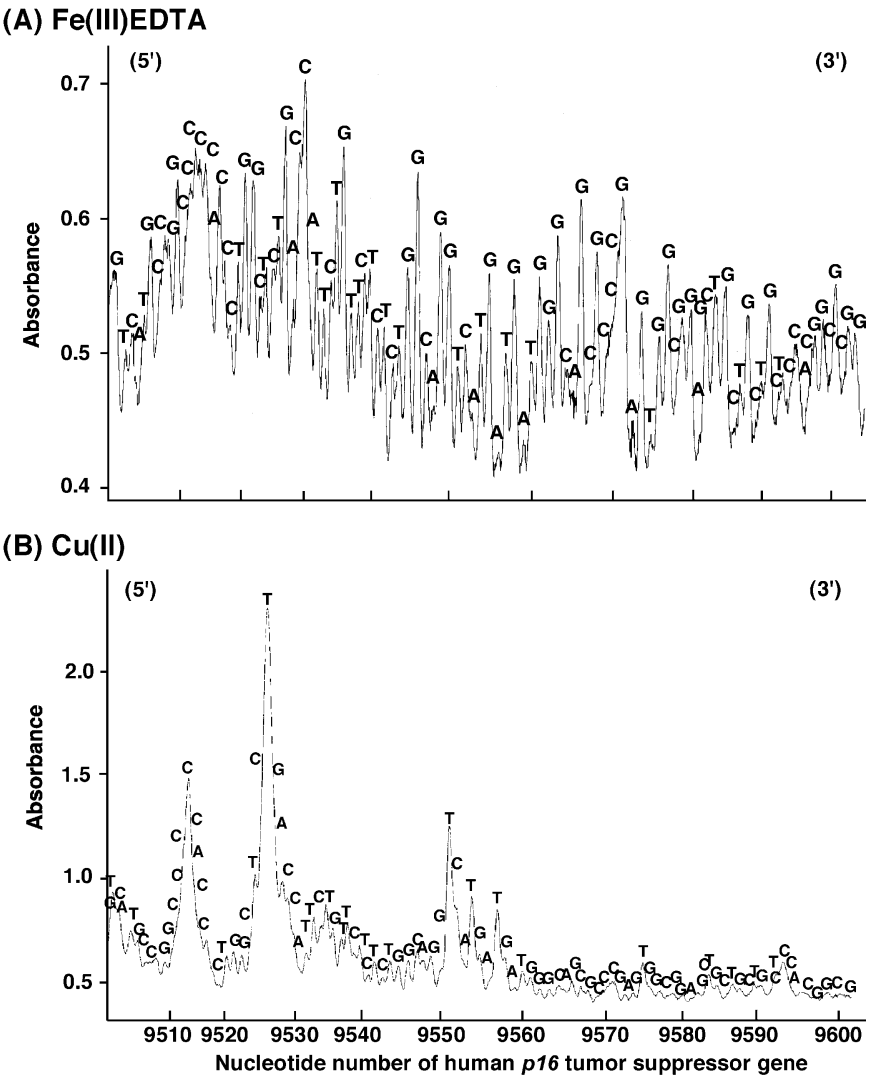

Figure 5. Site specificity of DNA damage induced by 6-OHDA in the presence of Fe(III)EDTA or Cu(II). Reaction mixtures contained the ^{32}P-labelled 309-base pair DNA fragments, 20 μM/base of calf thymus DNA, 20 μM (A) or 10 μM (B) 6-OHDA and 20 μM Fe(III)EDTA (A) or $CuCl_2$ (B) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated at 37°C for 1 h. Following piperidine treatment, the DNA fragments were analysed as described in Materials and methods.

formation of semiquinone radical of 6-OHDA. During the auto-oxidation, the M^n is reduced to M^{n-1} with concomitant generation of superoxide anion ($O_2^{\bullet -}$) from O_2, and subsequently $O_2^{\bullet -}$ is dismutated to H_2O_2. The semiquinone radical reacts with O_2 to generate $O_2^{\bullet -}$ and further autoxidize to *p*-quinone form. Fe(III)EDTA-mediated DNA damage resulting from exposure to 6-OHDA is caused by $^{\bullet}OH$ generated from the reaction of H_2O_2 with Fe(II) (Fenton reaction). DNA damage induced by 6-OHDA in the presence of Cu(II) is caused by the interaction of Cu(I) and H_2O_2 to form a Cu(I)-hydroperoxo complex.

6-OHDA is used to produce PD animal models through inducing selectively dopaminergic neuronal cell death. Iron and copper levels were increased in substantia nigra of rat treated with 6-OHDA [42–44], suggesting that iron and copper may exacerbate 6-OHDA neurotoxicity. In the present study, we demonstrated that iron and copper play a critical role in 6-OHDA-induced intracellular oxidative DNA damage leading to apoptosis. Accumulating evidence revealed that the imbalance in metal ion homeostasis is related to the aetiology of PD [45,46]. Some studies reported that lactoferrin, iron uptake protein, was up-regulated in PD, suggesting elevation of iron level [47,48]. It is reported that copper content increased in substantia nigra in PD with ageing [49]. These reports suggest accumulation of iron and copper in the brain of PD patients. This study raises the possibility that metal-mediated DNA damage may be involved in dopaminergic neuronal cell death in PD patients.

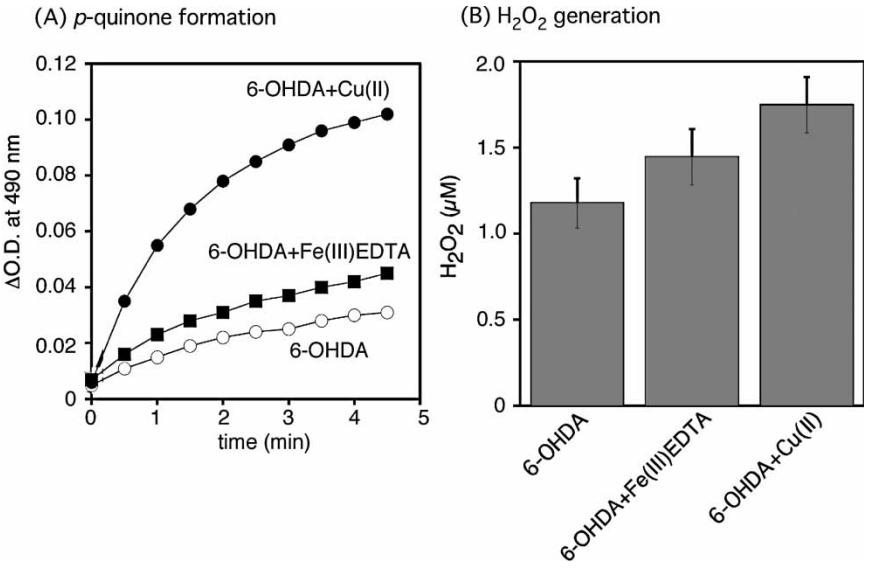

Figure 6. Effects of Fe(III)EDTA or Cu(II) on 6-OHDA auto-oxidation and H_2O_2 generation. (A) Reaction mixtures containing 5 μM DTPA was kept at 37°C and spectrum were measured immediately after addition of 500 μM 6-OHDA in the absence or presence of 20 μM metal ion. Thereafter, spectral tracing was repeated every 30 s for 270 s. The increments of absorbance at 490 nm (ΔO.D.) were plotted against the reaction time. (B) The amounts of H_2O_2 were measured by using scopoletin procedure. The reaction mixtures containing 10 μM 6-OHDA and 5 μM DTPA with or without 20 μM metal ions were incubated at 37°C for 5 min. Results are expressed as mean ± SE of values obtained from three independent experiments.

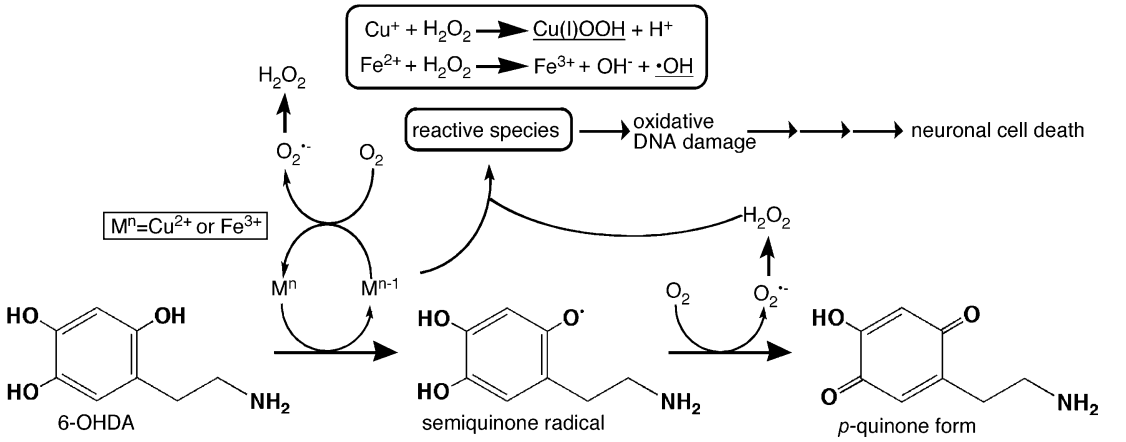

Figure 7. Possible mechanism of metal mediated DNA damage induced by 6-OHDA.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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