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Effect of Bromocriptine on 6-Hydroxydopamine-induced Lipid Peroxidation and Cytotoxicity *in vitro* and *in vivo*

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The present study was to evaluate the protective effects of bromocriptine, which is known as D_2 dopamine receptor agonist and used for the treatment of patients with Parkinson's disease (PD), on 6-hydroxydopamine (6-OHDA)-induced neurotoxicity *in vitro* and *in vivo*. Lipid peroxidation product (malondialdehyde; MDA) produced by the administration of 6-OHDA was profoundly reduced following the treatment of bromocriptine in a dose-dependent manner in rabbit brain homogenate. Quinone formation by 6-OHDA autoxidation was also attenuated, and its effect was as potent as other antioxidants. Pretreatment of bromocriptine reduced the cytotoxicity of 6-OHDA on SH-SY5Y neuroblastoma cell lines dose-dependently. The loss of striatal dopamine and its metabolite, DOPAC (dihydroxyphenylacetic acid) as well as increase of MDA production caused by intrastriatal injection of 6-OHDA was significantly recovered following the treatment of bromocriptine. The present study clearly showed that bromocriptine had a protective action against 6-OHDA-induced neurotoxicity. These results suggest that bromocriptine has the antioxidant properties, which could be another advantage for delaying the progress of Parkinson's disease.

Key Words: Bromocriptine, 6-OHDA, Lipid peroxidation, Antioxidant, Parkinson's disease

INTRODUCTION

The progressive neurodegeneration of nigrostriatal dopaminergic pathway is one of the principal factors accounting for the appearance of motor dysfunctions in Parkinson's disease (PD). Though the cause of PD remains unanswered, several evidences strongly suggest the involvement of oxidative stress.

Many neurotoxins can be converted into free radicals or produce free radicals(Chacon et al, 1987). Dopamine, which is produced from dopaminergic neurons in substantia nigra, can produce oxygen radicals by way of its metabolism or autoxidation. Both *in vitro* and *in vivo* studies showed oxidative stress-related neurotoxicity of 1-methyl-4-phenylpyridinium (MPP^+), an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) on dopaminergic neurons (Naoi et al, 1993; Langston et al, 1983). In addition, increased deposition of iron, which promotes the production of free radicals, and decrease in the levels of enzymes of the free radical scavenging system (Ambani et al, 1975; Kish et al, 1985) have been reported in the brain of patients with PD. The free radical hypothesis is therefore considered plausible for the pathogenesis of PD.

Levodopa was successfully used to compensate dopamine deficiency of patients with PD and improved parkinsonian motor deficits, presumably through its conversion to dopamine. However, long-term therapy using levodopa leads to a loss of drug efficacy and various problems like wearing-off, on-off phenomenon, dyskinesia, and psychotic symptoms (Przuntek et al, 1992; Silverman, 1993; Leiberman, 1994).

As an alternative to levodopa, dopamine (DA) re-

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ceptor agonists, such as pergolide, bromocriptine, lisuride, and apomorphine improve the symptoms of patients with PD (Ogawa, 1994). These drugs were shown to be more selective than levodopa in activating the dopamine receptor subtypes, and their effects last longer than that of levodopa.

Among various dopamine receptor agonists, bromocriptine (2-bromo-α-ergocriptine) is administered as monotherapy or combined therapy with levodopa for the treatment of PD patients(Calne et al, 1974; Kartzinel et al, 1976; Goldstein et al, 1978). Bromocriptine is a well known dopamine D_2 receptor agonist and widely used in the treatment of PD. In general, bromocriptine as well as some of D_2 receptor agonists attenuate the DA turnover rate through autoreceptor activation and are expected to protect the DA neurons by reducing the generation of dopamine-derived oxygen radicals. Recently, bromocriptine was reported to show superoxide and hydroxyl radical scavenging effect and inhibitory effects on lipid peroxidation of rat brain homogenate(Yoshikawa et al, 1994).

The present study was conducted to determine whether bromocriptine as an antioxidant has any beneficial effects on delaying the progress of Parkinson's disease by preventing or delaying 6-OHDA-induced damage *in vitro* and *in vivo*.

METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium), MEM (minimun essential medium) was purchased at Gibco (BRL Life Technologies, Inc. NY, USA). Bromocriptine, glucose, pyruvate, glutamine, penicillin, streptomycin, HEPES, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue], polyethylenimine (PEI), HBSS (Hanks' balanced salt solution), PBS (phosphate buffered saline) was purchased at Sigma Chem. Co.(St. Louis, MO. USA). 6-OHDA was purchased from RBI (Natick, MA. USA) and trolox, PBN (N-tert-butyl-α-phenylnitrone) was from Aldrich Chem. Co. (Milwaukee, WI. USA). Culture plate was from Falcon (Franklin Lakes, NJ. USA) and HPLC(high performance liquid chromatography) related materials was from Millipore Co. (Bedford, MA. USA). Other chemicals and reagents were of the purest grade available.

Lipid peroxidation in brain homogenate

Membrane fraction was obtained from rabbit striatum. Striatal tissue was immediately collected after New Zealand white rabbits (3~4 kg) were sacrificed. Striatal tissues was homogenized with ice cold homogenizing buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$ and 10 mM HEPES, pH 7.5) with Polytron homogenizer. The homogenate was centrifuged at 2800 rpm for 15 minutes. Pellet was resuspended in the same buffer and recentrifuged. After centrifugation, pellet was resuspended in this homogenating buffer and stored at −70°C until used. Protein was determined by Bradford method (Bradford et al, 1976).

Lipid peroxidation was measured by using thiobarbituric acid (TBA) method (Bilack & Tappel, 1973). Brain homogenate (2 mg/ml) was incubated with 200 μM 6-OHDA in the presence and absence of bromocriptine for 1 hour at 37°C in a water bath. For measurement of MDA, 300 μl of homogenate tissue was mixed with cocktail solution containing dH_2O, 8.1% of SDS, 20% acetic acid, 0.67% thiobarbituric acid. It was then shaken vigorously and boiled for 1 hour. After cooled with tap water, it was centrifuged at 3,000 rpm for 10 min at room temperature. Supernatants were used to calculate the amount of MDA levels by measuring absorbance at the 532 nm wavelength. MDA levels were expressed as nmol/mg protein.

Autoxidation of 6-OHDA

The formation of quinoidal product, 1,4-para-quinone by autoxidation of 6-OHDA was measured by monitoring absorbance change at 490 nm with a Spectrophotometer(Hitachi Ltd., 557, Japan)(Graham, 1978). 6-OHDA was prepared in 0.01N HCl. The reaction was initiated by the addition of 400 μM 6-OHDA at room temperature into cuvette. To check the effect of bromocriptine on the autoxidation of 6-OHDA, bromocriptine was added with 6-OHDA. Catalase, other antioxidants like trolox or PBN and hydroxyl radical scavengers such as mannitol or formate were also studied.

Cytotoxicity of 6-OHDA on neuronal cell line

SH-SY5Y, a catecholaminergic cell line, was cultured in DMEM medium supplemented with 10%

heat-inactivated fetal bovine serum (FBS), 2 mM pyruvate, 2 mM glutamine, 0.5% glucose, 50 μg/ml penicillin, 50 μg/ml streptomycin, 20 mM HEPES. Cells were maintained in a humidified 5% CO_2 atmosphere and was dissociated in late log phase with 0.05% trypsin. Cells were seeded at a density of 10,000/well in a polyethylenimine(PEI)-coated 96 well culture plate. 2 days after culture, medium was changed to MEM with 1% FBS and 6-OHDA was treated. 6-OHDA (20 mM stock) was dissolved in 0.01N HCl and bromocriptine (100 mM stock) was dissolved in 100% dimethylsulfoxide (DMSO) and diluted to an adequate concentration with MEM. Bromocriptine, trolox or PBN were added 1 hour before 6-OHDA treatment and maintained until cell viability was tested. 18 hours after treatment of 6-OHDA, MTT test was performed (Vistica et al, 1991). MTT (1 mg/ml) was added to each well and incubated for 3 hours. Then, culture medium was removed and each well was washed twice with PBS. After adding 100 μl of DMSO, absorbance at 540 nm was measured with a ELISA reader (Dynatech, MR700, USA).

Toxicity of intrastriatal 6-OHDA injection

Male Sprauge-Dawley rats(180~200 g) were provided with standard rat chow and used on stereotaxic surgery. Rats were anesthetized with equithesin (3 ml/kg) and placed in a stereotaxic instrument (Kopf, Tujunga, USA). A small hole was drilled in the skull and 2 μl of 6-OHDA (6 μg/μl 6-OHDA in saline containing 0.02% ascorbic acid) was slowely injected stereotaxically through a microsyringe in the right striatum. Lesion coordinates were AP+0.7 mm from bregma, ML 2.8 mm, DV 4.6 mm below the dura, with the incisor bar 3.3 mm below the internural line according to the Atlas of Paxinos and Watson (Paxinos & Watson, 1986). Control animals recieved unilateral injection of 0.02% ascorbic acid dissolved in 2 μl saline. Rats were injected intraperitoneally with either 0.3 or 3 mg/kg of bromocriptine daily for 7 days. In this experiment administration of bromocriptine was started 3 days before striatal injection of 6-OHDA and continued for 4 days after lesion making with 6-OHDA.

Rats were sacrificed by cervical dislocation 1 week after lesion making with 6-OHDA. Brain was immediately removed and striata were isolated and were stored at $-70^{\circ}C$ until measured. Lipid peroxidation of striatal tissue was measured by the same method described previously. For the measurement of DA and DOPAC, 300 μl of homogenated sample was centrifuged at 9,000 rpm for 30 min, the supernatant was filtered(0.2 μm diameter, Millipore) and equal volume of mixed solution containing 0.2 M perchloric acid, 0.2 mM EDTA, and dihydroxybenzylamine as an internal standard was added. Tissue concentration of DA and DOPAC was measured by high-performance liquid chromatography with an electrochemical detector (TOA electronics Ltd., ICA-5000, Japan). The amount of DA and DOPAC was expressed as nmol/mg protein.

Statistics

Data are presented as mean±SEM. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) tests and multiple comparison t-test to compare the difference using Sigmastat software (Jandel Co.).

RESULTS

Inhibition of lipid peroxidation by bromocriptine in rabbit brain homogenate

Lipid peroxidation product (MDA) caused by treatment of 6-OHDA in rabbit brain homogenate was measured. In untreated control, MDA production was 2.57±0.12 nmol/mg protein while 200 μM 6-OHDA significantly increase MDA production ($P<0.05$). However, treatment of bromocriptine significantly decreased MDA formation in a dose-dependent manner up to 800 μM (Fig. 1).

Inhibition of 6-OHDA autoxidation by bromocriptine

To evaluate the effect of bromocriptine on autoxidation of 6-OHDA, the absorbance of quinone formed by 6-OHDA autoxidation was measured spectrophotometrically at 490 nm. At 400 μM 6-OHDA 1,4-para-quinone was continuously formed, and autoxidation rate was 0.47±0.02 O.D./min. However, in the presence of bromocriptine, autoxidation rate of 6-OHDA was reduced in a dose dependent manner (Table 1). Antioxidants like trolox and PBN also attenuated the quinone formation. Catalase was less effective than bromocriptine, while hydroxyl radical scavengers like mannitol and formate did not show

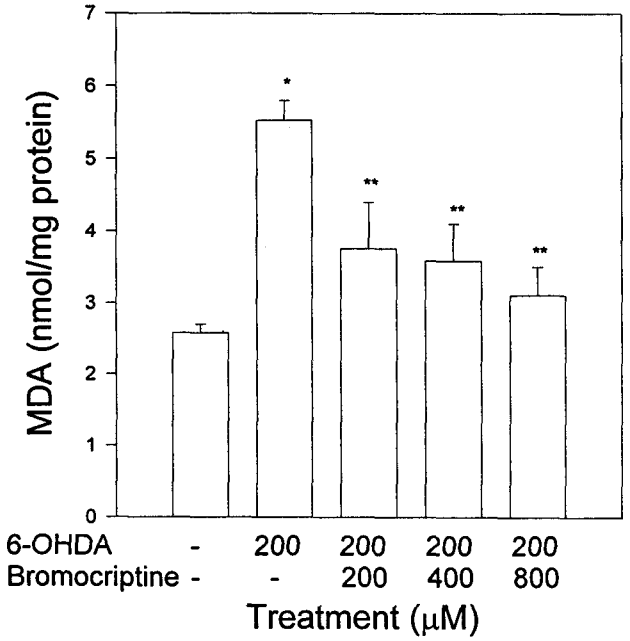

Fig. 1. Inhibitory effect of bromocriptine on lipid peroxidation induced by 6-hydroxydopamine in rabbit brain homogenate. 2 mg/ml of rabbit brain homogenate was incubated with 200 μM 6-OHDA in the presence and absence of bromocriptine for 1 hour at 37°C. Lipid peroxidation was estimated from production of malondialdehyde as described in Methods. Results are the mean± SEM for 4 experiments. *Significantly different from control ($P<0.05$); **Significantly different from that of 6-OHDA treated ($P<0.05$).

any significant inhibitory effect on 6-OHDA autoxidation (Table 2).

Protective effect of bromocriptine on 6-OHDA-induced cytotoxicity

To evaluate the cytotoxic effect of 6-OHDA on SH-SY5Y cell line, 6-OHDA was treated for 18 hours, and cell viability was measured by MTT assay. The treatment of 0.2 mM 6-OHDA induced significant cell death (35.1±3.5% compared to control). Pretreatment of bromocriptine dose-dependently reduced 6-OHDA-induced cytotoxicity. Trolox and PBN also showed the protective effect in 6-OHDA-induced cytotoxicity. When compared bromocriptine with these antioxidants, bromocriptine showed the most potent in protecting 6-OHDA-induced cell death (Fig. 2).

Table 1. Effects of bromocriptine on autoxidation of 6-OHDA

Treatment	Autoxidation rate (⊿O.D./min)	% of control
6-OHDA 400 μM	0.47±0.01	100
+ 50 μM Bromocriptine	0.38±0.01	81±3
+100 μM "	0.32±0.04	68±8
+200 μM "	0.27±0.02	58±4
+400 μM "	0.24±0.02	50±4

Autoxidation of 6-OHDA was monitored by the production of 1,4-para-quinone at 490 nm spectrophotometrically. Rate of autoxidation was determined as the initial absorbance change at unit time in the presence and absence of bromocriptine. Values are represented as the percentage of control rate. Results are the mean±SEM for 5 experiments.

Table 2. Comparisons of bromocriptine, antioxidants, hydroxyl radical scavengers and catalase on autoxidation of 6-OHDA

Addition	Autoxidation rate (⊿O.D./min)	% of control
6-OHDA 400 μM	0.47±0.02	100±4
+Bromocriptine 400 μM	0.24±0.02*	50±4
+Trolox 400 μM	0.23±0.02*	48±4
+PBN 400 μM	0.26±0.02*	56±4
+Mannitol 10 mM	0.46±0.05	97±11
+Formate 10 mM	0.55±0.06*	117±14
+Catalase 50 μg/ml	0.33±0.01	17±1

Experimental conditions were the same as Table 1 and described in Methods. Results are the mean±SEM for 5 experiments. *Significantly different from 6-OHDA treated ($P<0.05$).

Lipid peroxidation and biochemical changes induced by 6-OHDA injection into striatum

Bromocriptine (0.3 and 3 mg/kg) was treated for 3 days before and 4 days after intrastriatal injection of 6-OHDA. 1 week after 6-OHDA lesion making, rats were sacrificed and striatal tissue was homogenated. Lipid peroxidation and content of dopamine and DOPAC were measured to determine the 6-OHDA-induced neuronal damage. Lipid peroxidation in lesioned striatum was increased up to 127% com-

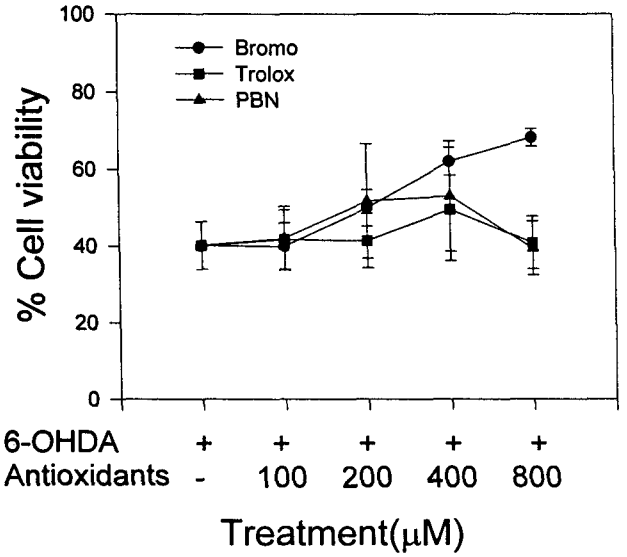

Fig. 2. Comparison of bromocriptine, trolox and PBN on 6-OHDA-induced cytotoxicity on neuroblastoma cell line, SH-SY5Y. 200 μM 6-OHDA was treated on SH-SY5Y neuroblastoma cells for 18 hours at 37°C. Bromocriptine, trolox and PBN were pretreated 1 hour before treatment of 6-OHDA and maintained until cell viability was tested. Cell viability was determined by using MTT reduction as described in Methods. Data are mean ± SEM for 8 experiments.

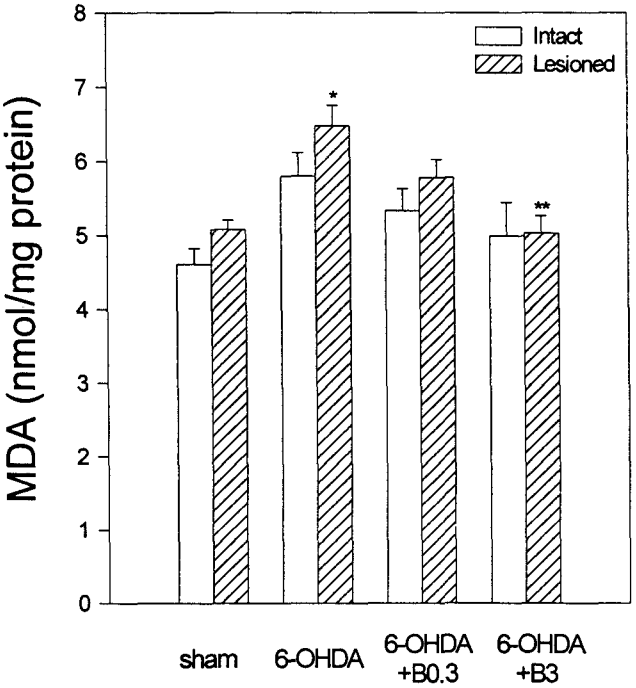

Fig. 3. Effect of bromocriptine on striatal lipid peroxidation in 6-OHDA-lesioned rats. Bromocriptine (0.3 or 3 mg/kg, ip) was administered daily 3 days before 6-OHDA-induced lesion making. The next day animals continuously received bromocriptine once a day for 4 days. Then 1 week after 6-OHDA lesion, striatal tissue were collected immediately after animals were sacrificed. Lipid peroxidation in striatal homogenate was determined from MDA production as described in Methods. B 0.3 and B3 represent 0.3, 3 mg/kg of bromocriptine treatment (ip) respectively. Results are the mean ± SEM for 9 experiments. *Significantly different from sham operated ($P < 0.05$); **Significantly different from 6-OHDA treated ($P < 0.05$).

pared with that of sham-operated animals (Fig. 3). Accompanying the elevation of MDA, injection of neurotoxin, 6-OHDA also resulted in the depletion of DA (27% of contralateral side) and DOPAC (41% of contralateral side) (Fig. 4 & 5).

As control, sham-operation did not significantly alter any of the above parameters. In contrast, however, treatment of 3 mg/kg bromocriptine reduced MDA level in lesioned striatum to the level of sham-ed striatum. DA and DOPAC content in lesioned striatum was recovered by bromocriptine. In addition higher dose of bromocriptine (3 mg/kg) showed more significant protection in 6-OHDA-lesioned striatal damage.

DISCUSSION

The present study demonstrates that 6-OHDA autoxidize to para-quinone and induce lipid peroxidation and cytotoxicity in SH-SY5Y neuroblastoma cell line, as confirmed by a marked elevation of lipid peroxidation product (malondialdehyde) and MTT reduction assay. In addition, intrastriatal administration of 6-OHDA also produced oxidative damage in lesioned striatum, leading to an increase in lipid peroxidation and the profound reduction in striatal dopamine content and DOPAC in lesioned striatum.

The results of the present experiments also reveal that 6-OHDA-induced cytotoxicity in neuroblastoma cell and oxidative neuronal damage in striatum can be prevented by the treatment of bromocriptine, as determined by the reduction of MDA levels and the recovery of dopamine content to the level of sham.

The selective toxicity of 6-OHDA for catecholaminergic nerve cells *in vitro* and *in vivo* is extensively studied for many years. It is generally accepted that free radicals are involved in 6-OHDA-

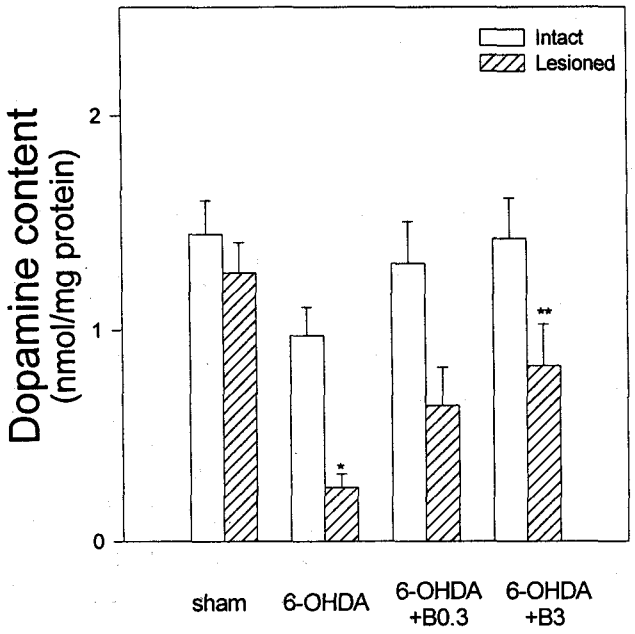

Fig. 4. Effect of bromocriptine on striatal dopamine content in 6-OHDA-lesioned rats. Administration of bromocriptine and striatal preparation were same as described in Fig. 3. Abbreviations and statistics were also same as in Fig. 3. Dopamine content was determined by HPLC with electrochemical detector. Results are the mean ± SEM for 8 experiments. *Significantly different from sham operated ($P<0.05$); **Significantly different from 6-OHDA treated ($P<0.05$).

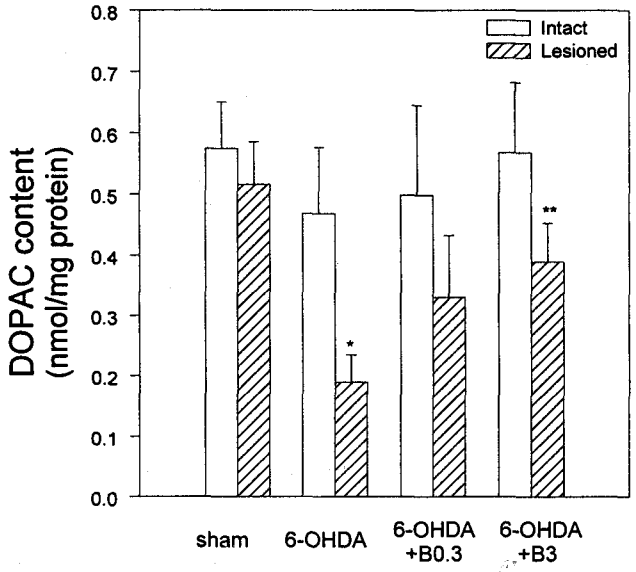

Fig. 5. Effect of Bromocriptine on striatal DOPAC content in 6-OHDA-lesioned rats. Experimental conditions and abbreviations were exactly same as in described in Fig. 4. DOPAC content was determined by HPLC with electrochemical detector. Results are the mean ± SEM for 8 experiments. *Significantly different from sham operated ($P<0.05$); **Significantly different from 6-OHDA treated ($P<0.05$).

induced toxicity (Tiffany-Castiglioni et al, 1982; Cohen, 1984; Kumar et al, 1995). 6-OHDA spontaneously reacts with molecular oxygen to form a 1,4-para-quinone, as well as oxygen radicals(Langston et al, 1983). The quinones generated by 6-OHDA oxidation bind covalently with sulfhydryl or other nucleophilic groups, leading to the inactivation of essential macromolecules. In addition, during the autoxidation of 6-OHDA, reactive oxygen species were formed and then initiated cell destruction.

These studies are in agreement with our present results of increase in malondialdehyde levels in rabbit brain homogenate and in lesioned striatum following intrastriatal injection of 6-OHDA.

An increasing number of evidences have suggested the involvement of active oxygen species or free radicals in various neurodegenerative disease, including PD (Ichitani et al, 1994; Schulz & Beal, 1995). Similarly, a number of works demonstrate that neurotoxicity of 6-OHDA is mediated by the oxidative stress, leading to the formation of free radicals and finally cell death in animal model of Parkinson's disease produced by neurotoxin, 6-OHDA. In this regard, a variety of antioxidants were applied in both *in vitro* and *in vivo* experiments for the protection of 6-OHDA-induced oxidative damage (Nakao et al, 1996; Kim et al, 1998).

Bromocriptine is a well known dopamine D_2 receptor agonist and widely used in the treatment of PD. In general, bromocriptine as well as some of D_2 receptor agonists attenuate the DA turnover rate through autoreceptor activation and are expected to protect the DA neurons by reducing the generation of dopamine-derived oxygen radicals. Recently, Yoshikawa et al. (1994) demonstrated, that, by using electron spin resonance (ESR) spectrophotometer, bromocriptine scavenged superoxide anion and hydroxyl radicals and had potent antioxidative activity.

In this study, we confirmed that bromocriptine significantly reduced 6-OHDA-induced increase in lipid peroxidation and autoxidation. Bromocriptine also exhibited the recovery of 6-OHDA-induced cytotoxicity and DA loss in lesioned striatum following intrastriatal injection of 6-OHDA. These results clearly show bromocriptine can act as an antioxidant

possibly by scavenging free radicals. It also strongly support previous reports that bromocriptine prevented a decrease in striatal DA loss induced by injection of 6-OHDA and desipramine, noradrenergic transporter inhibitor in mice (Asanuma et al, 1995) and delayed neuronal death of hippocampal neurons following cerebral ischemia in the gerbil (Liu et al, 1995).

There are some clinical reports which support a protective effect of bromocriptine. The incidence of side effects such as dyskinesia and on-off phenomena, after 5 years of treatment reduced markedly when bromocriptine was used in the early stage of PD, even if levodopa was added later (Bilack, 1973). Also, there were some reports that some dopamine agonists such as bromocriptine or pergolide not only have therapeutic effects in PD, but also show neuroprotective action in experimental models of free radical activity and normal aging.

Taken together with our present results and other previous reports, the therapeutic effects of bromocriptine on patients with PD may be partially due to its free radical scavenging activity or antioxidant action.

In conclusion, we showed that bromocriptine protects 6-OHDA-induced lipid peroxidation and neurotoxicity *in vitro* and *in vivo*. Considering a potent antioxidative action of bromocriptine demonstrated *in vitro*, the neuroprotective effects of bromocriptine *in vivo* may be, at least, due to its free radical scavenging activity.

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