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Differential Effect of Harmalol and Deprenyl on Dopamine-Induced Mitochondrial Membrane Permeability Change in PC12 Cells

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Abstract – Opening of the mitochondrial permeability transition pore has been recognized to be involved in cell death. The present study investigated the effect of β-carbolines (harmaline and harmalol) and deprenyl on the dopamine-induced change in the mitochondrial membrane permeability and cell death in differentiated PC12 cells. Cell death due to 250 μM dopamine was inhibited by caspase inhibitors (z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) and antioxidants (N-acetylcysteine, ascorbate, superoxide dismutase, catalase and carboxy-PTIO). β-Carbolines prevented the dopamine-induced cell death in PC12 cells, while deprenyl did not inhibit cell death. β-Carbolines decreased the condensation and fragmentation of nuclei caused by dopamine in PC12 cells. β-Carbolines inhibited the decrease in mitochondrial transmembrane potential, cytochrome c release, formation of reactive oxygen species and depletion of GSH caused by dopamine in PC12 cells, whereas deprenyl did not decrease dopamine-induced mitochondrial damage. β-Carbolines, deprenyl and antioxidants depressed the formations of nitric oxide and melanin in dopamine-treated PC12 cells. The results suggest that cell death due to dopamine in PC12 cells is mediated by caspase-8, -9 and -3. Unlike deprenyl, β-carbolines may attenuate the dopamine-induced cell death in PC12 cells by suppressing change in the mitochondrial membrane permeability through inhibition of the toxic action of reactive oxygen and nitrogen species.

Keywords □ β-carbolines, deprenyl, dopamine, mitochondrial membrane permeability, differentiated PC12 cells

INTRODUCTION

Mitochondrial dysfunction has been shown to participate in the induction of apoptotic and necrotic cell death (Chakraborati *et al.*, 1999). Defects in cellular respiration lead to diminished ATP production, increased sensitivity to oxidative attack and eventually cell death. The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis (Bernardi, 1999; Cassarino *et al.*, 1999; Chakraborati *et al.*, 1999). Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, releases of Ca^{2+} and cytochrome c, osmotic swelling and loss of oxidative phosphorylation. The permeability transition pore is suggested as target of the dopamine oxidation products and 1-methyl-4-phenylpyridinium (MPP^+) (Berman and Hastings, 1999; Kim *et al.*, 1999; Lee *et al.* 2002). The oxidation of dopamine liberates free radicals and dopamine quinone, which causes a swelling of isolated brain mitochondria and loss of the mitochondrial transmembrane potential. The mitochondrial membrane permeability transition due to dopamine is attenuated by thiol compounds and antioxidants (Offen *et al.*, 1996; Lee *et al.*, 2002).

Deprenyl, an inhibitor of monoamine oxidase (MAO)-B, provides a beneficial effect in the treatment of Parkinson's disease (Birkmayer *et al.*, 1985). However, the action mechanism by which deprenyl exerts a neuroprotective effect is uncertain. Deprenyl shows a differential effect on neuronal cells against neurotoxins. Deprenyl does not decrease the cytotoxic effect of dopamine on chick telencephalic neurons (Jacobsson and Fowler, 1999) and rather enhances the dopamine-induced cytotoxicity in neuroblastoma SH-SY5Y cells (Lai and Yu, 1997). Deprenyl and pargyline show a little or no protective effect on mouse hippocampal cell lines against glutamate toxicity (Maher and Davis, 1996). It has been reported that deprenyl provides a protective effect on nigral neurons against MPP^+ toxicity independent of the inhibition of MAO-B (Wu *et al.* 2000).

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1-Methlated β-carbolines have been postulated to act as endogenous neurotoxins because of their structural similarity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Albores *et al.,* 1990; Gearhart *et al.,* 2002). The compounds at high concentrations are reported to induce tremor and learning impairment. These compounds inhibit activities of MAO-A and -B (Fuller *et al.*, 1986). Harmaline reveals a cytotoxic effect on PC12 cells (Cobuzzi *et al.*, 1994) and causes a degeneration of Purkinje cells in rat cerebellum (O'Hearn and Molliver, 1993). In contrast, it has demonstrated that β-carbolines exert a protective effect against the toxic action of some neurotoxins. Coadministration of harmalol inhibits glutamate- or MPP^+-induced cell death in neuronal cells (Maher and Davis, 1996; Park *et al.*, 2003).

Despite inhibition of cell viability loss, the effect of 1-methylated β-carbolines on change in the mitochondrial membrane permeability due to dopamine, followed by viability loss in cells, has not been clarified. In addition, β-carbolines and deprenyl have been suggested to show a differential effect on toxic cell injuries depending on cell types. The purpose of the present study was to explore cytotoxicity of dopamine in relation to mitochondrial dysfunction. Thus, the effect of β-carbolines (harmaline and harmalol) and deprenyl on differentiated PC12 cells against toxicity of dopamine was assessed by measuring the effect on the change in transmembrane potential, cytochrome c release, formation of reactive oxygen species (ROS), GSH contents, caspase-3 activity and cell viability.

MATERIALS AND METHODS

Materials

Harmaline (1-methyl-7-methoxy-3,4-dihydro-β-carboline), harmalol (3,4-dihydro-1-methyl-9H-pyrido[3,4-b]indol-7-ol), 3-hydroxytyramine (dopamine, DA), superoxide dismutase (SOD, from bovine erythrocytes), catalase (from bovine liver), N-acetylcysteine, carboxy-PTIO, rutin trihydrate, z-Ile-Glu-(O-ME)-Thr-Asp(OMe) fluoromethyl ketone (z-IETD.fmk), z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD.fmk). z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (z-DQMD.fmk), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, rhodamine 123, 2,7-dichlorofluorescin diacetate ($DCFH_2$-DA), glutathione (GSH, reduced form), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, phenylmethylsulfonylfluoride (PMSF), nerve growth factor 7S (NGF 7S), 2-deoxy-D-ribose, 2,4-dinitrophenylhydrazine and RPMI 1640 medium were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Quantikine M rat/mouse cytochrome c assay kit was obtained from R&D systems (Minneapolis, MN, USA), ApoAlertTM CPP32/caspase-3 assay kit from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), fetal bovine serum (FBS) from Life Technologies (GibcoBRL, Grand Island, NY, USA).

Cell Culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, South Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin as described in the manual of the cell line bank. Cells were differentiated by treating with 100 ng/mL 7S NGF in the 60 × 15 mm cell culture dishes coated with poly-L-lysine (10 μg/mL) for 9 days (Tatton *et al.*, 2002). Cells were washed with RPMI medium containing 1% FBS 24 h before experiments and replated onto the 96 and 24 well plates.

Cell Viability Assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). PC12 cells (4×10^4) were treated with dopamine in the presence of β-carbolines for 24 h at 37°C. The medium (200 μL) was incubated with 10 μL of 10 mg/mL MTT solution for 2 h at 37°C. Culture medium was removed, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340, Sunnyvale, CA, USA). Cell viability was expressed as a percent of the control culture value.

Morphological Observation of Nuclear Damage

PC12 cells (1×10^6 cells/mL) were treated with dopamine in the presence of β-carbolines for 24 h at 37°C, and the nuclear morphological change was assessed using Hoechst dye 33258 (Oberhammer *et al.*, 1992; Lotharius *et al.*, 1999). Cells were incubated with 1 μg/mL Hoechst 33258 for 3 min at room temperature, and nuclei were visualized using an Olympus Microscope with a WU excitation filter (Tokyo, Japan).

Measurement of Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential was measured using the dye rhodamine 123 (Fu *et al.*, 1998). It has been shown that the uptake of rhodamine 123 into mitochondria is a function of mitochondrial transmembrane potential. PC12 cells (4×10^4)

were incubated with dopamine for the indicated time and then were incubated for 20 min at 37°C in RPMI containing 10 μM rhodamine 123. Cell suspension was centrifuged at 412 g for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

Measurement of Cytochrome c Release

Cytochrome c released into the cytosol of PC12 cells was assessed by using a solid phase ELISA kit for the detection of rodent cytochrome c. The cells (5×10^5/mL) harvested by centrifugation at 412 g for 10 min were washed twice with PBS and resuspended in 250 mM sucrose, 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol and 0.1 mM PMSF. The cells were further homogenized by successive passages through a 26-gauge needle. The homogenates were centrifuged at 100,000 g for 30 min. The supernatant obtained was used for analysis of cytochrome c. The supernatants were added into the 96 well microplates coated with monoclonal antibody specific for rat/mouse cytochrome c that contain cytochrome c conjugate. The procedure was performed as described in the assay kit. Absorbance of samples was measured at 450 nm in a microplate reader. Like samples, the diluted solutions of cytochrome c standard were added to the microplates coated with monoclonal antibody, and the standard curve was constructed. The amount was expressed as nanograms/mL by using a standard curve.

Measurement of Caspase-3 Activity

Apoptosis in PC12 cells was assessed by measuring activity of caspase-3 that is considered to be involved in programmed cell death (Chandra *et al.*, 2000). Cells (2×10^6 cells/mL) were treated with dopamine in the presence of β-carbolines for 24 h at 37°C. The effect of β-carbolines on apoptosis in the dopamine-treated cells was determined as described in users manual of ApoAlertTM CPP32/caspase-3 assay kit. The supernatant obtained by a centrifugation of dissolved cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and was incubated for 1 h at 37°C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from absorbances in the *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as 1 nmol of chromophore *p*-nitroanilide produced.

Measurement of Intracellular ROS Formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu *et al.*, 1998). After exposure to dopamine, PC12 cells (4×10^4) were incubated with 50 μM dye for 30 min at 37°C and were then washed with PBS. The cell suspensions were centrifuged at 412 g for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

Measurement of Total Glutathione

The total glutathione (GSH + GSSG) was determined using glutathione reductase (van Klaveren *et al.*, 1997). PC12 cells (1×10^5/mL) were treated with dopamine for 24 h at 37°C. The cell suspensions were centrifuged at 412 g for 10 min in a microplate centrifuge, and medium was removed. Cells were dissolved with 2% 5-sulfosalicylic acid (100 μL) and then incubated in 100 μL of the solution containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37°C. Twenty microliters of glutathione reductase (100 U/mL) was added to the mixture, which was further incubated for 10 min. Absorbance was measured at 412 nm using a microplate reader.

Measurement of Nitrite/Nitrate Production

Nitric oxide liberated from the oxidation of dopamine in PC12 cells was measured by assaying nitric oxide metabolites, nitrite and nitrate (NO_x) (Kim *et al.*, 2001). PC12 cells (4×10^4) were incubated with 500 μM dopamine in the presence of β-carbolines for 24 h at 37°C. Nitrate in the medium was reduced to nitrite by incubation with nitrate reductase (500 mU/mL, 160 μM NADPH and 4 μM flavin adenine dinucleotide at room temperature for 2 h. The medium was mixed with an equal amount of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% phosphoric acid). Absorbance was measured at 550 nm, and the amount of nitrite produced was determined using sodium nitrite as the standard. The results were expressed as total nitrite equivalents (NO_x).

Measurement of Dopamine-Melanin Product

Dopamine-melanin, the end product of dopamine oxidation,

was measured as described in the previous reports (Linert *et al.*, 1996; Lai and Yu, 1997). The reaction mixture contained 4 × 10^4 PC12 cells, 250 μM dopamine and RPMI, and reaction was performed for 4 h at 37°C. Changes in absorbance due to the dopamine-melanin were measured at 405 nm. The amount of melanin formed was assayed using a commercial melanin (oxidation product of tyrosine) as the standard.

Statistical Analysis

The values given in the text are expressed as the means ± SEM. The data were analyzed by one-way analysis of variance. The analysis of variance justified post hoc comparisons between the different groups by using the Duncan's test. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of β-Carbolines on the Cell Viability Loss in PC12 Cells Treated with Dopamine

The dopamine-induced cell death was assessed using the PC12 cells differentiated by NGF. When PC12 cells were treated with 250 μM dopamine for 24 h, the cell viability decreased to 47% in the MTT assay. The cytotoxic effect of dopamine was inhibited by 40 μM IETD.fmk (a cell permeable inhibitor of caspase-8), 40 μM LEHD.fmk (a cell permeable inhibitor of caspase-9) and 40 μM DQMD.fmk (a cell permeable inhibitor of caspase-3) and various antioxidants [1 mM N-acetylcysteine, 1 mM ascorbate (a scavenger of ROS and reactive nitrogen species), 10 μg/mL SOD (a scavenger of superoxide anion, 10 μg/mL catalase (a scavenger of hydrogen peroxide) and 25 μM carboxy-PTIO (a specific scavenger of nitric oxide)] (Fig. 1A). The concentrations of β-carbolines and deprenyl used in this study were based on the previous reports (Maher and Davis, 1996; Park *et al.*, 2003). Harmaline and harmalol (25 μM) attenuated the dopamine-induced viability loss of PC12 cells, whereas 25 μM deprenyl did not show an inhibitory effect (Fig. 1B). Because β-carbolines are suggested to act as a neurotoxin, the present study investigated the cytotoxic effect of compound alone on PC12 cells. Harmaline, harmalol and deprenyl (25 μM) for a 24 h-incubation did not cause a significant loss of cell viability in PC12 cells.

To clarify the inhibitory effect of β-carbolines against cytotoxicity of dopamine, the present study investigated the effect on the nuclear morphological changes observed in the dopamine-treated cells. Nuclear staining with Hoechst 33258 demonstrated that control PC12 cells had regular and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei characteristic of apoptotic cells were evident in PC12 cells treated with 250 μM dopamine for 24 h at 37°C (Fig. 2). Harmalol (25 μM) decreased the dopamine-induced nuclear damage while the nuclear morphology in cells exposed to harmalol alone was similar to that in the control cells.

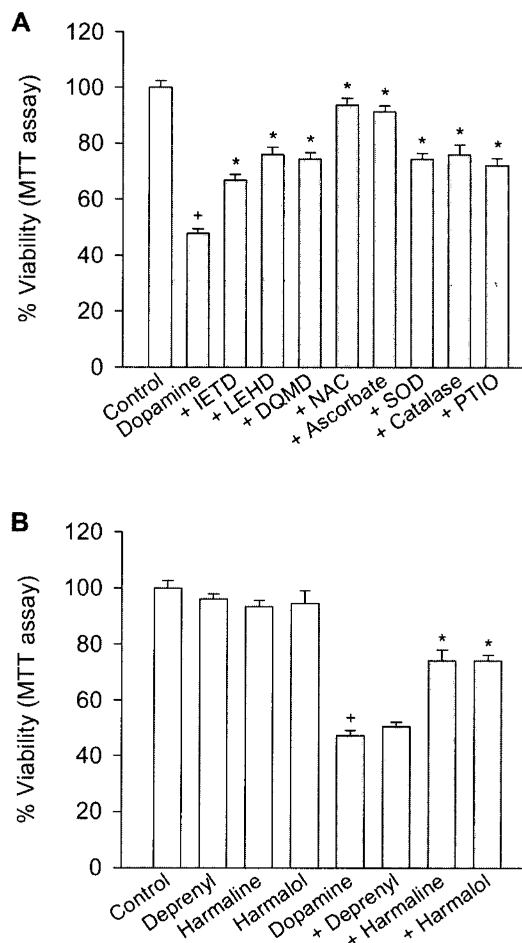

Fig. 1. Effect of β-carbolines and deprenyl on the dopamine-induced loss of cell viability. PC12 cells (4 × 10^4) were treated either with 250 μM dopamine in the presence of caspase inhibitors (40 μM of z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) and various antioxidants [1 mM N-acetylcysteine (NAC), 1 mM ascorbate, 10 μg/mL SOD, 10 μg/mL catalase and 25 μM carboxy-PTIO] (A) or with dopamine in the presence of 25 μM harmaline, 25 μM harmalol or 25 μM deprenyl (B) for 24 h at 37°C. After this, the mixtures were treated with 0.5 mg/mL MTT for 2 h. Data are expressed as the percentage of control and represent the means ± SEM of 6 replicate values in two separate experiments. $+p<0.05$, compared to control. $*p<0.05$, compared to dopamine alone.

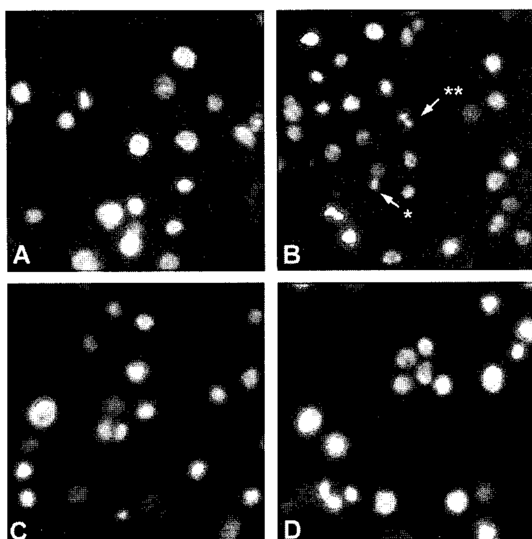

Fig. 2. Effect of β-carbolines on apoptotic cell death induced by dopamine. PC12 cells (1×10^6 cells) were treated with 250 μM dopamine in the presence of 25 μM harmalol for 24 h at 37°C. The cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. (A) control cells; (B) cells treated with dopamine alone; (C) cells treated with dopamine and 25 μM harmalol; (D) cells treated with harmalol alone. Arrow indicates condensed (*) and fragmented (**) nuclei. All the subparts are representative of four different experiments.

Effect of β-Carbolines on Transmembrane Potential, Cytochrome c Release, Activation of Caspase-3, ROS Formation, and GSH Depletion in PC12 Cells

Disruption of the mitochondrial transmembrane potential and a subsequent cytochrome c release have been recognized to be implicated in apoptosis (Bernadi, 1999). Measuring the effect on the mitochondrial membrane potential we assessed the cytotoxic effect of dopamine. Change in the mitochondrial transmembrane potential in PC12 cells treated with dopamine was quantified by measuring the rhodamine 123 uptake. When PC12 cells were treated with 250 μM dopamine for 4 h at 37°C, the rhodamine 123 uptake decreased. β-Carbolines (25 μM) and 10 μg/mL antioxidant enzymes prevented the dopamine-induced increase in the rhodamine 123 uptake, whereas deprenyl did not attenuate it (Fig. 3A). β-Carbolines and deprenyl alone did not significantly decrease the rhodamine 123 uptake.

The release of cytochrome c from mitochondria to the cytosol due to loss of the mitochondrial membrane potential causes the activation of caspase-3 that is involved in apoptotic cell death (Chandra *et al.*, 2000). The dopamine-induced change in the mitochondrial membrane permeability was assessed by measuring a release of cytochrome c into the cytosol. PC12 cells treated with 250 μM dopamine for 4 h revealed a significant increase in cytochrome c release (Fig. 3B). Harmaline and harmalol (25 μM) significantly decreased the dopamine-induced release of cytochrome c, while β-carbolines alone and 25 μM deprenyl did not significantly release cytochrome c.

Apoptosis in PC12 cells was assessed by measuring the activity of caspase-3 (Du et al., 1997). Treatment of PC12 cells with 250 μM dopamine for 24 h revealed an increase in caspase-3 activity, which was depressed by 10 μg/mL of SOD and catalase. Addition of harmaline and harmalol (25 μM) attenuated the activation of caspase-3 due to dopamine in PC12 cells (Fig. 3C). β-Carbolines alone (25 μM) increased the caspase-3 activity in PC12 cells by 8-11%, but these values were not significantly different from the control.

To determine whether ROS are involved in the dopamine-induced cell death in PC12 cells, the formation of ROS within cells was measured by monitoring a conversion of $DCFH_2$-DA to DCF. Exposure of PC12 cells to 250 μM dopamine for 24 h caused a significant increase in DCF fluorescence, which was significantly depressed by 25 μM of harmaline or harmalol but not decreased by 25 μM deprenyl (Fig. 4A). β-Carbolines and deprenyl alone did not significantly increase the DCF fluorescence.

Drops in GSH levels increase the sensitivity of neurons to the toxic effect of neurotoxins (Zeevalk *et al.*, 1997) and are associated with mitochondrial dysfunction (Pereira and Oliveira, 2000). The present study investigated the effect of β-carbolines on the dopamine-induced decrease in GSH contents. The GSH content in the control PC12 cells was 6.57 ± 0.19 nmol/1×10^5 cells. Treatment of 250 μM dopamine showed the decrease in GSH contents (33%), which was depressed by 25 μM harmaline, 25 μM harmalol and 10 μg/mL antioxidant enzymes (SOD and catalase). Meanwhile, the decrease in GSH contents due to dopamine was not significantly inhibited by deprenyl (Fig. 4B). β-Carbolines and deprenyl alone (25 μM) did not decrease the GSH contents in PC12 cells.

Effects of β-Carbolines on Nitric Oxide and Melanin Liberated from Dopamine Oxidation

The inhibitory effect of antioxidants on the dopamine-induced cell death and the formation of ROS suggest that the cytotoxic effect of dopamine is mediated by oxidative stress. Particularly, the inhibitory effect of N-acetylcysteine, ascorbate and carboxy-PTIO suggests involvement of reactive nitrogen species in the cytotoxicity of dopamine. The present study investi-

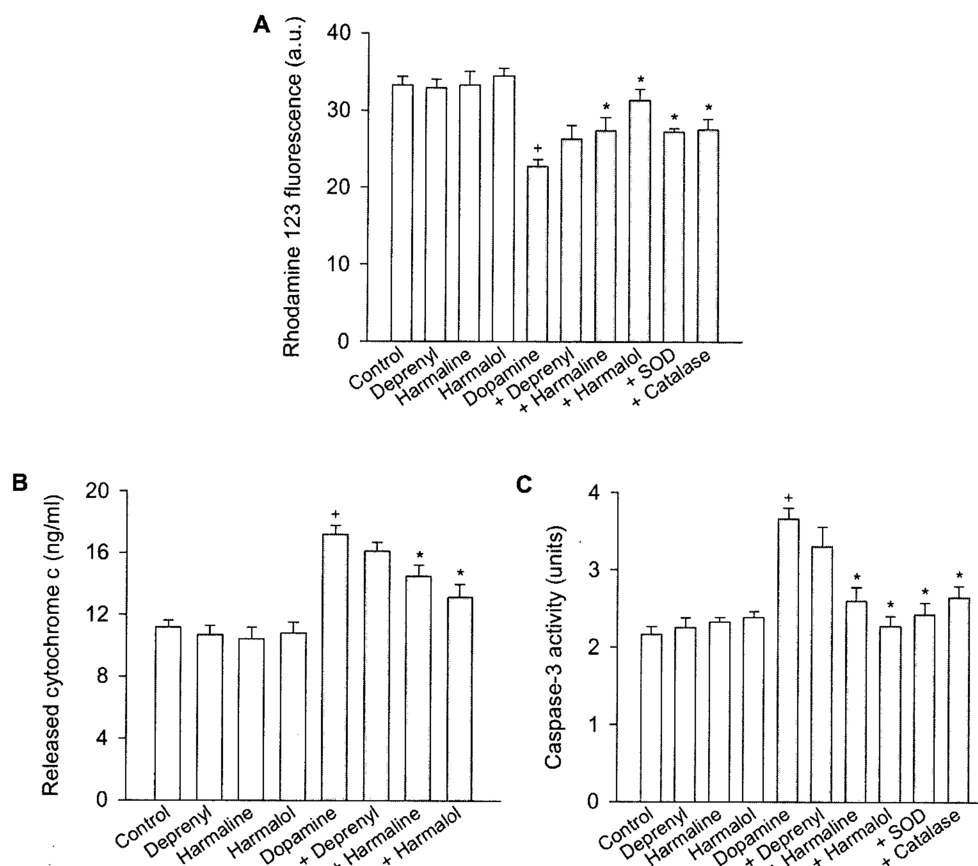

Fig. 3. Effect of β-carbolines on the loss of mitochondrial membrane potential, release of cytochrome c and activation of caspase-3 due to dopamine. PC12 cells were treated with 250 μM dopamine in the presence of 25 μM β-carbolines, 25 μM deprenyl and 10 μg/mL antioxidant enzymes (SOD and catalase). In the assay of the mitochondrial membrane potential (A), 4×10^4 PC12 cells were treated with dopamine for 4 h at 37°C, in the assay of the cytochrome c release (B), 5×10^5 PC12 cells were treated with dopamine for 4 h, and in the assay of caspase-3 activity (C), 2×10^6 PC12 cells were treated with dopamine for 24 h. The values are expressed as arbitrary units of fluorescence in the mitochondrial membrane potential, as nanograms/mL in the cytochrome c and as units in the caspase-3 activity. Data represent the means ± SEM of 5-6 replicate values in two separate experiments. +$p<0.05$, significantly different from control. *$p<0.05$, significantly different from dopamine alone.

gated the decomposing effect of β-carbolines on the reactive nitrogen species or melanin liberated from the oxidation of dopamine in PC12 cells. Incubation of dopamine for 24 h at 37°C in the medium containing 4×10^4 PC12 cells produced 6.43 μM of NO_x. β-Carbolines (5 and 25 μM) and scavengers of reactive nitrogen species (1 mM N-acetylcysteine and 50 μM rutin) decreased production of nitric oxide due to oxidation of dopamine (250 μM), whereas 25 μM deprenyl did not significantly attenuate it (Fig. 5A).

Incubation of 250 μM dopamine in the reaction mixture containing 4×10^4 PC12 cells for 4 h produced about 24.0 μg of melanin (Fig. 5B). The formation of melanin due to the dopamine oxidation was decreased by the addition of 25 μM harmaline, 25 μM deprenyl, 1 mM N-acetylcysteine (an inhibitor of melanin formation) and 10 μg/mL of antioxidant enzymes (SOD and catalase), while the inhibitory effect of harmalol on the melanin formation was not seen.

DISCUSSION

The pathologic feature in Parkinson's disease shows the striking degenerative loss of dopaminergic neurons in the nigrostriatal system. Rat PC12 cells display phenotypic characteristics of both adrenal chromaffin cells and sympathetic neurons (Greene and Tischler, 1976). Upon NGF stimulation, PC12 cells differentiate into fully neurite-bearing, sympathetic neuron-like phenotype (Fujita *et al.*, 1989) and show increase in choline acetyltransferase expression (Kalisch *et al.*, 2002). In addition, PC12 cells exposed to NGF display abundant neuritic growth, produce dopamine and express dopamine transporter

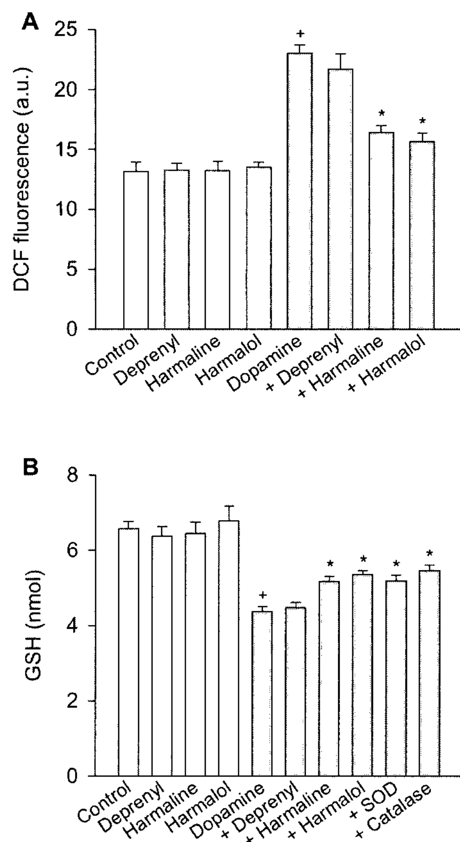

Fig. 4. Effect of β-carbolines on increased ROS formation and decrease in GSH contents due to dopamine. In the assay of ROS (A), 4×10^4 PC12 cells were treated with 250 μM dopamine in the presence of 25 μM β-carbolines or 25 μM deprenyl for 24 h at 37°C, and in the assay of GSH contents (B), 1×10^5 PC12 cells were treated with 250 μM dopamine in the presence of 25 μM β-carbolines, 25 μM deprenyl and 10 μg/mL antioxidant enzymes for 24 h. The values are expressed as arbitrary units of fluorescence in ROS formation and as nmol in GSH contents. Data represent the means ± SEM of 5-6 replicate values in two separate experiments. $+p<0.05$, significantly different from control. $*p<0.05$, significantly different from dopamine alone.

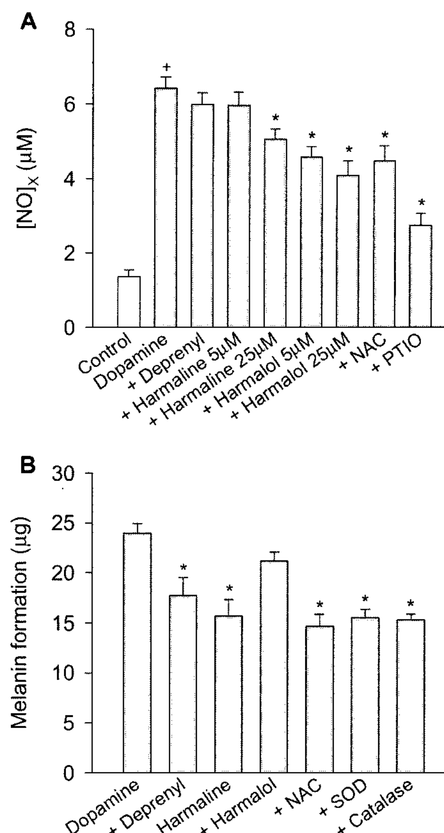

Fig. 5. Effect of β-carbolines on nitric oxide (NO_x) and melanin formed from oxidation of dopamine. PC12 cells (4×10^4) were incubated with 250 μM dopamine in the presence of compounds [25 μM β-carbolines, 1 mM N-acetylcysteine (NAC), 25 μM carboxy-PTIO, 10 μg/mL SOD and 10 μg/mL catalase]. In the assay of NO_x (A), PC12 cells were treated with dopamine for 24 h at 37°C, and in the assay of melanin (B), PC12 cells were treated with dopamine for 4 h. Data represent the means ± SEM of 6 replicate values in two separate experiments. $+p< 0.05$, significantly different from control. $*p<0.05$, significantly different from dopamine alone.

(Kadota *et al.*, 1996). Under the character of PC12 cells that reveals a neurochemical dopaminergic phenotype, the current study assessed the cytotoxicity of dopamine against dopaminergic neurons using the PC12 cells differentiated with NGF. Apoptotic cell death is suggested to be mediated by interaction of ligand with cell surface CD95 receptor followed by caspase-8 activation and by the mitochondrial membrane permeability change that results in the release of cytochrome c and subsequent activation of caspase-9 and -3 (Mignotte and Vayssiere, 1998). A significant cytotoxic effect of dopamine on cell viability in differentiated PC12 cells was demonstrated by using MTT assay and by observing nuclear morphological changes with Hoechst 33258 stain, which indicated necrotic and apoptotic cell death. The condensation and fragmentation of nuclei and a significant increase in caspase-3 activity indicated the apoptotic death in PC12 cells treated with dopamine. The inhibitory effect of specific caspase inhibitors (z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) suggests that the dopamine-induced apoptotic cell death in differentiated PC12 cells is mediated by activation of caspase-8 and by mitochondrial damage, leading to the release of cytochrome c and activation of caspase-9 and -3. These processes may cause nuclei damage and cell viability loss.

In a number of cells, there is evidence that mitochondrial dysfunction and increased formation of ROS, which may be

followed by depletion of GSH, are involved in cell death (Chandra *et al.*, 2000). The purpose of the present study was to explore the cytotoxicity of dopamine in relation to change in the mitochondrial membrane permeability. The toxic effect of dopamine on neuronal cells has been mediated by formation of free radicals and GSH depletion (Offen *et al.*, 1996; Lee *et al.* 2002). The oxidation of dopamine generates free radicals and toxic quinones. The cytoxicity of dopamine on neuronal cells appears to be mediated by the formation of ROS and reduction of cellular GSH contents (Offen *et al.*, 1996). The similar result was seen in the differentiated PC12 cells exposed to dopamine. The inhibitory effect of N-acetylcysteine, ascorbate, antioxidant enzymes and carboxy-PTIO suggests that change in the mitochondrial membrane permeability and release of cytochrome c due to dopamine in differentiated PC12 cells are mediated by increased formation of free radicals and depletion of GSH. Dopamine can undergo spontaneous autoxidation to form dopamine quinine and ROS, such as superoxide anoin and hydrogen peroxide (Graham, 1984). Thus, the depressant effect of exogenously added-antioxidant enzymes (SOD and catalase, cell impermeable antioxidant enzymes) postulates that the cytotoxic effect of dopamine on PC12 cells has been partially mediated by exogenous ROS.

Mitochondrial MAO-B is considered to play a part in the progress of nigrostriatal cell death. It has been proposed that deprenyl exerts a beneficial effect in the treatment of Parkinson's disease through a selective inhibition of MAO-B (Birkmayer *et al.*, 1985). In contrast, there is evidence that the protective effect of deprenyl against the toxic effect of neurotoxins may not be mediated by the MAO inhibition (Tatton and Chalmers-Redman, 1996; Wu *et al.*, 2000). It has been suggested that 1-methylated β-carbolines (inhibitors of MAO-A and -B) may act as neurotoxins (Matsubara *et al.*, 1998). However, in contrast to the low level of endogenous β-carbolines, the intraperitoneal administration of high concentrations (0.5 mmol/kg of norharman or 0.25 mmol/kg of 9-mono-N'-methylnorharman) is found to induce parkinsonism in mice (Matsubara *et al.*, 1998). 2-Methyl-harmalinium cations (250 μM) causes cell death in PC12 cells (Cobuzzi *et al.*, 1994). Thus, the results indicate that *in vivo* and *vitro* studies, high concentrations of β-carbolines are necessary to show a toxic effect. In this study, 25 μM of harmaline and harmalol did not exhibit a significant cytotoxicity on differentiated PC12 cells. As previously mentioned, β-carbolines attenuate the cytotoxicity of glutamate in HT-22 hippocampal cell line and that of MPP^+ in differentiated PC12 cells (Maher and Davis, 1996; Park *et al.*, 2003). However, in the respect of the mitochondrial membrane permeability the mechanism by which β-carbolines decrease the cytotoxicity of dopamine is uncertain. The present results suggest that harmaline and harmalol attenuate the dopamine-induced loss of mitochondrial transmembrane potential, cytochrome c release and activation of capase-3 in differentiated PC12 cells by suppressing the increased formation of ROS and by reversing the decrease in GSH contents. Inhibition of the mitochondrial membrane permeability change by β-carbolines probably decreases dopamine-induced cell death. Meanwhile, it is unlikely that suppression of the dopamine cytotoxicity due to β-carbolines is accomplished by inhibition of mitochondrial MAO activity. β-Carbolines reveal a non-selective inhibition on mitochondrial MAO (Fuller *et al.*, 1986). Deprenyl at high concentrations inhibits MAO-A as well as MAO-B (Tatton and Chalmers-Redman, 1996). However, deprenyl does not decrease the toxic effect of dopamine on chick telencephalic cells (Jacobsson and Fowler, 1999). In contrast to β-carbolines, the deprenyl did not decrease dopamine-induced change in the mitochondrial membrane permeability, followed by cell death, in differentiated PC12 cells. Thus, β-carbolines appear to depress the toxicity of dopamine against PC12 cells without intervention of MAO inhibition.

Free radical scavengers prevent the age-related increase in the protein carbonyl content in mouse synaptic mitochondria (Banaclocha *et al.*, 1997) and decrease apoptotic cell death in the dopaminergic cell lines and PC12 cells treated with MPP^+ (Seaton *et al.*, 1997). The present results have also suggested that β-carbolines attenuate the mitochondrial damage and cell death in PC12 cells that may be caused by oxidative stress. The enzymatic or non-enzymatic oxidation of dopamine produces free radicals and neuromelanin that may show a toxic effect on neuronal cells (Lai and Yu, 1997; Berman and Hastings, 1999; Offen *et al*, 1999). Neuromelanin is found to be phagocytised by neuronal cells, which may cause a cell death (Offen *et al.*, 1999). The present results suggest that the protective effect of β-carbolines on PC12 cells against cytotoxicity of dopamine appears to be mediated by inhibition of the formations of reactive nitrogen species melanin. However, dopamine-melanin seems to play a minor role in the cytotoxicity of dopamine, because 25 μM melanin for a 24 h treatment caused about 12% of cell death in PC12 cells in the MTT assay (data not shown).

Overall, the results suggest that cell viability loss due to dopamine in PC12 cells is mediated by caspase-8, -9 and -3. β-Carbolines may decrease the dopamine-induced cell death in PC12 cells by attenuating the mitochondrial membrane perme-

ability change through inhibition of the toxic action on reactive oxygen and nitrogen species. β-Carbolines appear to exert a protective effect on PC12 cells against the toxic action of dopamine through action(s) different from deprenyl.

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