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Inhibition of Glutamate-Induced Change in Mitochondrial Membrane Permeability in PC12 cells by 1-Methylated β-carbolines

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Abstract–1-Methylated β-carbolines (harmaline and harmalol) and antioxidants (N-acetylcysteine and ascorbate) reduced the loss of cell viability in differentiated PC12 cells treated with 5 mM glutamate. β-Carbolines prevented the glutamate-induced decrease in mitochondrial membrane potential, cytochrome c release and caspase-3 activation in PC12 cells. β-Carbolines reduced the formation of reactive oxygen species and depletion of glutathione due to glutamate in PC12 cells. β-Carbolines revealed a scavenging action on hydrogen peroxide and reduced the iron and EDTA-mediated degradation of 2-deoxy-D-ribose. The results suggest that 1-methylated β-carbolines attenuate the cytotoxic effect of glutamate on PC12 cells by reducing the alteration of mitochondrial membrane permeability that seems to be mediated by oxidative stress.

Keywords □ β-carbolines; glutamate; mitochondrial membrane permeability; PC12 cells

Mitochondrial dysfunction and increased oxidative stress are considered to be involved in the pathogenesis of neurodegenerative disorders, such as Parkinson's disease (Olanow and Tatton, 1999). Defect in mitochondrial function has been shown to participate in the induction of neuronal cell death (Bernadi, 1996; Lotharius *et al.*, 1999; Atlante *et al.*, 2001). The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of neuronal injury as well as apoptosis. Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, release of Ca^{2+} and cytochrome c, osmotic swelling and loss of oxidative phosphorylation (Bernardi, 1996; Cassarino *et al.*, 1999). The neuronal cell deaths induced by 6-hydroxydopamine, glutamate and MPP^+ (1-methyl-4-phenylpyridinium) are mediated by opening of permeability pore and collapse of the mitochondrial membrane potential (Chakraborati *et al.*, 1999; Lotharius *et al.*, 1999; Chandra *et al.*, 2000; Lee *et al.*, 2002).

1-Methylated 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) (Matsubara *et al.*, 1998; Gearhart *et al.*, 2002). The compounds at high concentrations are reported to induce tremor and learning impairment. 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 to these reports, β-carbolines are demonstrated to exhibit a protective effect against the damaging action of some neurotoxins. Co-administration of harmalol attenuates the increased activities of antioxidant enzymes and formation of tissue peroxidation products in the brain of mouse treated with MPTP (Lee *et al.*, 2000) and decreases the catecholamine-induced cell death in PC12 cells (Kim *et al.*, 2001).

The effect of β-carbolines on neuronal cells against the cytotoxic effect of glutamate has not been clearly elucidated in relation to the mitochondrial membrane permeability. Therefore, the present study examined the protective effect of β-carbolines (harmaline and harmalol) on PC12 cells against the cytotoxicity of glutamate by measuring the change in transmembrane potential, cytochrome c release, caspase-3 activity, formation of reactive oxygen species (ROS) and GSH contents.

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), monosodium L-glutamate, N-acetylcysteine, ascorbic acid, 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123, 2, 7-dichlorofluorescin diacetate ($DCFH_2$-DA),

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glutathione (GSH, reduced form), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, 2-deoxy-D-ribose, 2,4-dinitrophenylhydrazine, and RPMI 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), ApoAlert™ CPP32/caspase-3 assay kit from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), and 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 nerve growth factor 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 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 glutamate 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.

Measurement of apoptosis by a caspase-3 activity assay

Apoptosis in PC12 cells was assessed by measuring activity of caspase-3 (Chandra *et al.*, 2000). Cells (2×10^6 cells/mL) were treated with glutamate in the presence of β-carbolines for 24 h at 37°C The effect of β-carbolines on apoptosis in the glutamate-treated cells was determined as described in user's manual of ApoAlert™ CPP32/caspase-3 assay kit. The supernatant obtained by a centrifugation of cells dissolved 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 nM of chromophore *p*-nitroanilide produced.

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 glutamate for 4 h and then were incubated for 20 min at 37°C in DMEM 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 800 g for 10 min were washed twice with phosphate buffered saline (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. The amount was expressed as nanograms/mL by using a standard curve.

Measurement of ROS formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent 2,7-dichlorofluorescin (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu *et al.*, 1998). After exposure to glutamate, PC12 cells (4×10^4) were incubated with 50 μM dye for 30 min at 37°C and then were 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.

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 glutamate 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 hydrogen peroxide decomposition and 2-deoxy-D-ribose degradation

β-Carbolines was added to a reaction mixture containing 120 mM KCl, 0.1 mM H_2O_2, and 50 mM Tris-HCl, pH 7.4. The reaction was performed for 30 min and terminated by addition of a stopping solution (25 mg/mL potassium biphthalate, 2.5 mg/mL NaOH, 82.5 mg/mL potassium iodide and 0.25 mg/mL ammonium molybdate) (Lee *et al.*, 2000). The hydrogen peroxide remained was determined using H_2O_2 solution as the standard.

The decomposing effect of β-carbolines on hydroxyl radicals was determined by an assay of malondialdehyde chromogen formation due to 2-deoxy-D-ribose degradation (Aruoma *et al.*, 1994). The reaction mixtures contained 2 mM 2-deoxy-D-ribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, 150 mM KCl, and 50 mM NaH_2PO_4 buffer, pH 7.4. After a 30-min incubation, the reaction was stopped by adding 1% thiobarbituric acid in 50 mM NaOH and 2.8% trichloroacetic acid. Absorbance was measured at 532 nm.

Statistical analysis

The values given in the text are expressed as the means ± SEM values. 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 Duncans test. A probability less than 0.05 was considered to be statistically significant.

RESULTS

Effect of ß-carbolines on the cell viability loss and caspase-3 activity in PC12 cells treated with glutamate

The effect of β-carbolines on the glutamate-induced cell death was assessed using the PC12 cells that were differentiated by nerve growth factor. When PC12 cells were treated with 5 mM glutamate for 24 h, cell viability decreased to 40%. The present study examined the involvement of ROS in the cytotoxic effect of glutamate. Addition of 1 mM N-acetylcysteine and 1 mM ascorbate (scavengers of ROS and reactive nitrogen species) reduced the glutamate-induced cell death. β-Carbolines depressed the glutamate-induced viability loss of PC12 cells, and the effect of β-carbolines reached to a plateau at 25-50 μM (Fig. 1). Thus, at 25 μM we assessed the effect of β-carbolines against the cytotoxic effect of glutamate. β-Carbolines (25 μM) exhibited 24-26% of protective effect, which was less than the effect of N-acetylcysteine and ascorbate (86 and 89%). β-Carbolines alone for a 24 h-incubation did not significantly affect cell viability of PC12 cells.

Loss of the mitochondrial transmembrane potential may be involved in apoptotic cell death (Bernardi, 1996; Lotharius *et al.*, 1999). Apoptosis in PC12 cells was assessed by measuring the activity of caspase-3 (Du *et al.*, 1997). Treatment of PC12 cells with 5 mM glutamate for 24 h revealed an increase in caspase-3 activity in PC12 cells, which was depressed by 25

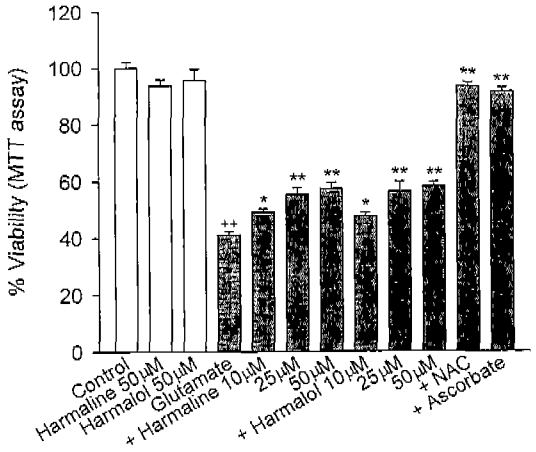

Fig. 1. Effect of β-carbolines on the glutamate-induced loss of cell viability. PC12 cells (4×10^4) were treated with 5 mM glutamate in the presence of β-carbolines and antioxidants (1 mM N-acetylcysteine [NAC] and 1 mM ascorbate) for 24 h at 37°C, and then mixtures were treated with 0.5 mg/mL MTT for 2 h. The values are expressed as the percentage of control and are the means ± SEM ($n = 9$). $^{++}p < 0.01$, significantly different from the control. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from glutamate alone.

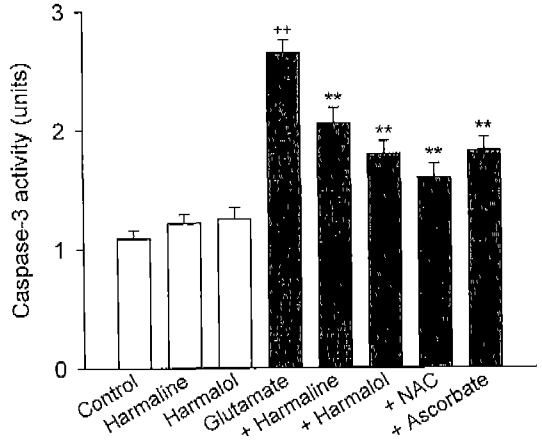

Fig. 2. Effect of β-carbolines on the glutamate-induced increase in caspase-3 activity. PC12 cells (2×10^6) were treated with 5 mM glutamate in the presence of β-carbolines (25 μM), 1 mM N-acetylcysteine (NAC) and 1 mM ascorbate for 24 h at 37°C. Caspase-3 activity was measured using the assay kit. Each point is the means ± SEM (n = 6). $^{++}p < 0.01$, significantly different from the control. $^{**}p < 0.01$, significantly different from glutamate alone.

μM of β-carbolines, 1 mM N-acetylcysteine and 1 mM ascorbate (Fig. 2). β-Carbolines alone did not affect caspase-3 activity in PC12 cells.

Effect of β-carbolines on mitochondrial transmembrane potential, cytochrome c release, ROS formation and GSH depletion in PC12 cells

Disruption of the mitochondrial transmembrane potential has been recognized to be implicated in apoptosis (Bernardi, 1996). Measuring the effect on the mitochondrial transmembrane potential assessed the cytotoxic effect of glutamate. Change in the mitochondrial transmembrane potential in PC12 cells treated with glutamate was quantified by measuring the cellular retention of rhodamine 123. When PC12 cells were treated with 5 mM glutamate for 4 h at 37°C, a decrease in the retention of rhodamine 123 was observed. β-Carbolines (25 μM) prevented the glutamate-induced decrease in the retention of rhodamine 123 (Fig. 3). Loss of the mitochondrial membrane potential causes the release of cytochrome c from mitochondria to the cytosol, which is followed by the activation of caspase-3 (Chandra *et al.*, 2000). The glutamate-induced change in the mitochondrial membrane permeability was assessed by measuring a release of cytochrome c into the cytosol. The PC12 cells incubated with 5 mM glutamate for 4 h revealed a significant increase in the level of cytochrome c in the cytosol (Fig. 3). β-Carbolines (25 μM) significantly reduced the glutamate-induced release of cytochrome c. The

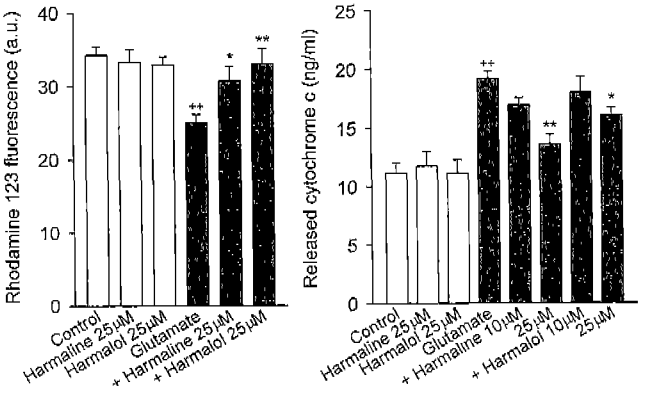

Fig. 3. Effect of β-carbolines on change in the mitochondrial transmembrane potential and on cytochrome c release due to glutamate. PC12 cells (4×10^4 in assay of the mitochondrial transmembrane potential and 5×10^5 in cytochrome c release) were treated with 5 mM glutamate and β-carbolines for 4 h at 37°C. The uptake of rhodamine 123 reflects the mitochondrial transmembrane potential, which are expressed as arbitrary units of fluorescence. The values are the means ± SEM (n = 4-5). $^{++}p < 0.01$, significantly different from the control. $^{*}p < 0.05$ and $^{**}p < 0.01$, significantly different from glutamate alone.

compound alone did not significantly stimulate release of cytochrome c.

To determine whether ROS were involved in the glutamate-induced cell death in PC12 cells, we investigated the formation of ROS within cells by monitoring a conversion of $DCFH_2$-DA to DCF. Exposure of PC12 cells to 5 mM glutamate for 24 h caused a significant increase in DCF fluorescence. β-Carbo-

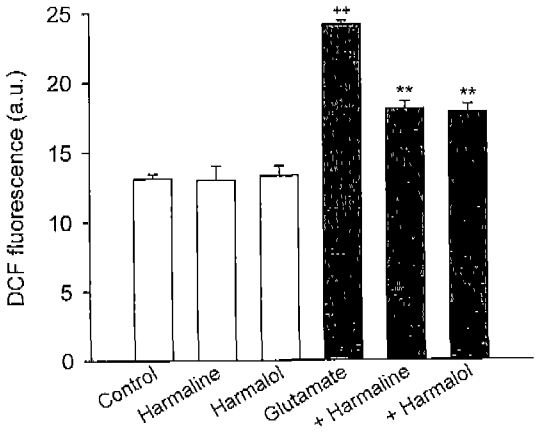

Fig. 4. Effect of β-carbolines on the formation of ROS due to glutamate. PC12 cells (4×10^4) were treated with 5 mM glutamate and 25 μM β-carbolines for 24 h at 37°C, and then mixtures were treated with 50 μM $DCFH_2$-DA for 30 min. The values are expressed as arbitrary units of fluorescence, and each point is the means ± SEM (n = 4). $^{++}p < 0.01$, significantly different from the control. $^{**}p < 0.01$, significantly different from glutamate alone.

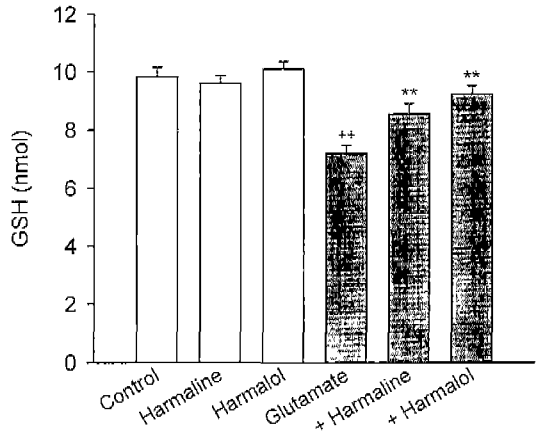

Fig. 5. Effect of β-carbolines on the glutamate-induced decrease in GSH contents. PC12 cells (1×10^5) were treated with 5 mM glutamate and 25 μM β-carbolines for 24 h at 37°C. The values are expressed as nmol, and each point is the means ± SEM (n = 6). $^{++}p < 0.01$, significantly different from the control. $^{**}p < 0.01$, significantly different from glutamate alone.

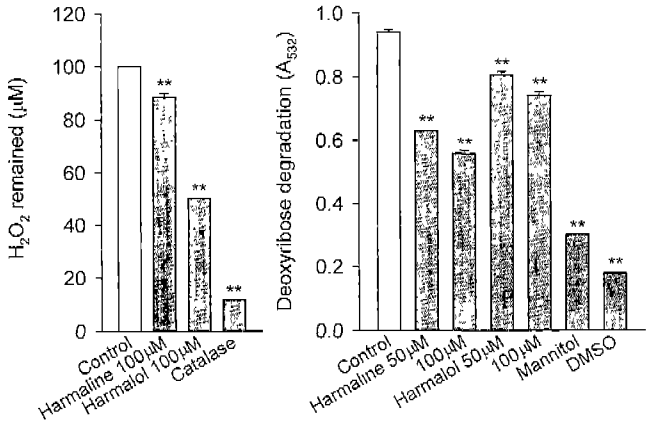

Fig. 6. Effect of β-carbolines on hydrogen peroxide decomposition and 2-deoxy-D-ribose degradation. β-Carbolines and oxidant scavengers (10 mM mannitol, 10 mM DMSO or 2.5 μg/mL catalase) were added to the reaction mixtures containing either 20 μM H_2O_2 or 2 mM 2-deoxy-D-ribose, 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate. The reaction was performed for 30 min. The values are means ± SEM (n = 5). $^{**}p<0.01$, significantly different from the control.

lines (25 μM) significantly depressed the glutamate-induced increase in DCF fluorescence (Fig. 4).

Reduction of GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins (Zeevalk *et al.*, 1997) and is associated with mitochondrial dysfunction (Pereira and Oliveira, 2000). In this study, we investigated the effect of β-carbolines on the glutamate-induced decrease in GSH contents. The GSH content in the control PC12 cells was 9.83±0.33 nmol/1×10^5 cells. Glutamate (5 mM) reduced the GSH contents by 28%, which was depressed by 25 μM of β-carbolines (Fig. 5). The compound alone did not significantly reduce the GSH contents in PC12 cells.

Decomposing effect of β-carbolines on ROS

The inhibitory effect of antioxidants on the glutamate-induced cell death and the formation of ROS suggest that the cytotoxic effect of glutamate may be mediated by oxidative stress. Hydrogen peroxide, a cell permeable oxidant, is produced by the dismutation of the superoxide anion and is regarded as a precursor of highly reactive oxidants (Halliwell and Gutteridge, 1999). The effect of β-carbolines on oxidative damage of PC12 cells was examined by measuring the decomposing effect on hydrogen peroxide. β-Carbolines and catalase (2.5 μg/mL) significantly decomposed hydrogen peroxide, and the effect of harmalol was greater than that of harmaline (Fig. 6).

The scavenging effect of β-carbolines on hydroxyl radicals was assayed by measuring the degradation of 2-deoxy-D-ribose produced by Fe^{3+}, EDTA, H_2O_2 and ascorbate (Aruoma, 1994). The 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate-induced deoxyribose degradation were significantly inhibited by 10 mM of hydroxyl radical scavengers (mannitol and DMSO). β-Carbolines (50 and 100 μM) significantly attenuated the iron and EDTA-mediated degradation of 2-deoxy-D-ribose (Fig. 6).

DISCUSSION

A significant cytotoxic effect of glutamate on cell viability in differentiated PC12 cells was demonstrated by the decrease in MTT conversion and the caspase-3 activation. Opening of the mitochondrial permeability transition pore causes the release of cytochrome c from mitochondria to the cytosol, leading to the activation of caspase-3 that is involved in apoptotic cell death (Amarante-Mendes *et al.*, 1998; Chandra *et al.*, 2000). A significant increase in caspase-3 activity revealed the apoptotic death in PC12 cells treated with glutamate.

The loss of the mitochondrial membrane potential and cytochrome c release were observed in the glutamate-treated PC12 cells. Reduction in mitochondrial GSH levels and concomitant increase in ROS are found during the apoptotic process in the immortalized mouse hippocampal cell line exposed to glutamate (Tan *et al.*, 1998). In PC12 cells depleted of GSH, the increased formation of ROS and a subsequent rise of Ca^{2+} level may cause cell death (Jurma *et al.*, 1997). The present result also suggests that the depletion of GSH may play a major

role in the cytotoxic effect of glutamate. The depressant effects of N-acetylcysteine and ascorbate postulate the involvement of ROS in the cytotoxic effect of glutamate on PC12 cells. In addition, the increased formation of ROS in PC12 cells suggests strongly that the change in the mitochondrial membrane permeability due to glutamate, followed by cell death, has been mediated by 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 Parkinsons disease through a selective inhibition of MAO-B (Birkmayer *et al.*, 1985). It has 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 ß-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 reveal a toxic effect. In this study, 50 μM of harmaline and harmalol did not exhibit a significant cytotoxicity on differentiated PC12 cells. The previous report has shown that harmalol and harmaline reduce the catecholamine-induced cell death in undifferentiated PC12 cells (Kim *et al.*, 2001). The present results suggest that β-carbolines may reduce the glutamate-induced cell death in differentiated PC12 cells by attenuating the loss of mitochondrial transmembrane potential, cytochrome c release, increased formation of ROS and decrease in GSH contents. Involvement of the MAO inhibition in the inhibitory effect of β-carbolines against the cytotoxicity of glutamate is probably excluded by the following results. Despite the evidence that oxidative stress due to the oxidation of dopamine may be involved in neurodegeneration (Olanow and Tatton, 1999), deprenyl does not reduce the toxic effect of dopamine on neuronal cells (Lai and Yu, 1997; Jacobsson and Fowler, 1999). There is evidence that the protective effect of deprenyl against the toxic effect of neurotoxins has not been mediated by the inhibition of MAO (Tatton and Chalmers-Redman, 1996; Wu *et al.*, 2000). Thus, β-carbolines appear to depress the toxicity of glutamate against PC12 cells without intervention of MAO inhibition.

The present results have suggested that β-carbolines attenuates the mitochondrial damage and cell death in PC12 cells that may be caused by oxidative stress. Thus, the protective effect of β-carbolines was assessed by observing the scavenging action on ROS. β-Carbolines revealed a scavenging action on hydrogen peroxide, a cell permeable oxidant. The degradation of 2-deoxy-D-ribose induced by Fe^{3+}, EDTA, H_2O_2 and ascorbate is used as a sensitive detection method to assay hydroxyl radicals and is decreased by addition of the hydroxyl radical scavengers mannitol, DMSO and sodium formate (Aruoma, 1994). The inhibitory effect of β-carbolines on the deoxyribose degradation suggests their scavenging action on hydroxyl radicals. On the basis of concentrations, β-carbolines exhibited a depressant effect on the deoxyribose degradation comparable to hydroxyl radical scavengers. The present result revealed that β-carbolines exerted an antioxidant effect.

In conclusion, the results show that β-carbolines may reduce the glutamate-induced viability loss in PC12 cells by attenuation of the mitochondrial membrane permeability change and by a scavenging action on ROS.

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