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Effects of Harmaline and Harmalol on Dopamine Quinone-induced Brain Mitochondrial Dysfunction

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Abstract–The present study elucidated the effect of β-carbolines (harmaline and harmalol) on brain mitochondrial dysfunction caused by the tyrosinase-induced oxidation of dopamine. Harmaline, harmalol and antioxidant enzymes (SOD and catalase) attenuated the dopamine-induced alteration of membrane potential, cytochrome c release and thiol oxidation in mitochondria. In contrast, antioxidant enzymes failed to reverse mitochondrial dysfunction induced by dopamine plus tyrosinase. β-Carbolines decreased the damaging effect of dopamine plus tyrosinase against mitochondria, except no effect of harmalol on thiol oxidation. Antioxidant enzymes decreased the melanin formation from dopamine in the reaction mixture containing mitochondria but did not reduce the formation of dopamine quinone caused by tyrosinase. Both harmalol and harmaline inhibited the formation of reactive quinone and melanin. Harmalol being more effective for quinone formation and vise versa. The results indicate that compared to MAO-induced dopamine oxidation, the toxic effect of dopamine in the presence of tyrosinase against mitochondria may be accomplished by the dopamine quinone and toxic substances other than reactive oxygen species.β-Carbolines may decrease the dopamine plus tyrosinase-induced brain mitochondrial dysfunction by inhibition of the formation of reactive quinone and the change in membrane permeability.

Key words □ β-carbolines, dopamine, tyrosinase, brain mitochondria, protection

Defect in mitochondrial function has been shown to participate in the induction of neuronal cell death (Bernadi, 1996; Lotharius *et al.*, 1999; Lee *et al.*, 2002a). The hydrogen peroxide, one of dopamine oxidation products, diffuses partly into the mitochondrial matrix and oxidizes glutathione to glutathione disulfide, which is attributed to induce the alteration of cellular functions, including suppression of the thiol-dependent electron transport (Cohen *et al.*, 1997). The major mitochondrial defect in Parkinson's disease appears to be associated with complex I at the electron transport chain (Olanow and Tatton, 1999). In addition, the membrane permeability transition of mitochondria is known to play a part in neuronal injury.

Neuronal cell death induced by mitochondrial complex I inhibitors and 1-methyl-4-phenylpyridinium (MPP^+) has been suggested to be mediated by the opening of permeability pore and the collapse of the mitochondrial membrane potential (Seaton *et al.*, 1997; Cassarino *et al.*, 1999; Lotharius *et al.*, 1999). The opening of mitochondrial permeability transition pore has been shown to induce depolarization of the transmembrane potential, release of cytochrome c, osmotic swelling and loss of oxidative phosphorylation (Bernardi, 1996; Berman and Hastings, 1999; Cassarino *et al.*, 1999). The permeability transition pore is suggested as another target of the dopamine oxidation products and MPP^+ in mitochondria (Kim *et al.*, 1999; Lee *et al.*, 2000; Lee *et al.*, 2002a). The oxidation of dopamine liberates reactive oxygen species and dopamine quinone. Dopamine quinone, an electron-deficient species, reacts with cellular macromolecules, which may show a neurotoxic effect (Stokes *et al.*, 1999). This species causes a significant increase in the swelling of brain and liver mitochondria (Berman and Hastings, 1999).

N-methylated β-carbolines, including harman and harmaline, have been shown to exert multiple pharmacological actions, such as monoamine oxidase (MAO) inhibition, convulsive or anticonvulsive action and anxiolytic effect (Loew *et al.*, 1985; Barbaccia *et al.*, 1986; Fernandez de Arriva *et al.*, 1994). The compounds, as endogenous neurotoxins, have been suggested to be involved in the pathogenesis of idiopathic Parkinson's disease (Albores *et al.*, 1990; O'Hearn and Molliver, 1993; Gearhart *et al.*, 2000). This suggestion may be supported by that the levels of norharman and harman in plasma and cerebrospinal fluid of patients with Parkinson's disease are found to be higher than in the control group (Kuhn *et al.*, 1995, 1996).

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However, several reports have indicated that β-carbolines show a protective effect on neuronal cells against neurotoxins. Harmine is demonstrated to attenuate the cytotoxic effect of glutamate on mouse hippocampal cells (Maher and Davis, 1996). We have shown that harmaline and harmalol revealed a protective effect on 1-methyl-4-phenyl-1,2,3,6-tetrahydrpyridine (MPTP)-induced neurotoxicity in the mouse (Lee *et al.*, 2000) and decreased the catecholamines-induced brain mitochondrial dysfunction and cell death in PC12 cells (Kim *et al.*, 2001; Lee *et al.*, 2002b).

Despite these findings, the effect of β-carbolines on mitochondrial function against toxic action of dopamine quinone has not been elucidated. In this respect, we elucidated effect of β-carbolines on the dopamine plus tyrosinase-induced mitochondrial dysfunction and compared it with that on the toxic action of dopamine alone. The present study examined the effects of β-carbolines (harmaline and harmalol) on the membrane potential, electron flow, cytochrome c release and thiol oxidation in brain mitochondria exposed to dopamine quinone.

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), tyrosinase (from mushroom), superoxide dismutase (from bovine erythrocytes; 2,500-7,000 units/mg of protein, SOD), catalase (from bovine liver; 10,000-25,000 units/mg of protein), rotenone, safranin O, antimycin A, myxothiazol, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 5,5'-dithio-bis-(2-nitrobenzoic acid)(DTNB) and melanin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA.). All other reagents were of analytical grade.

Preparation of brain mitochondria

Mitochondria were prepared from the brain (cerebrum) of male Sprague-Dawley rats weighing between 150 and 200 g according to the method of Clark and Nicklas (1970). The brain was removed and homogenized in 9 volume of ice-cold medium (250 mM sucrose, 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4). The homogenate was cleared by centrifugation at 2,000 g for 2 min, and then the supernatant was centrifuged at 12,500 g for 8 min. The mitochondrial pellet was resuspended in 10 ml of a 3% Ficoll medium (3% Ficoll, 120 mM mannitol, 30 mM sucrose and 25 μM EDTA, pH 7.4). The suspension was carefully layered onto 20 ml of a 6% Ficoll medium (6% Ficoll, 240 mM mannitol, 60 mM sucrose and 50 μM EDTA, pH 7.4) and centrifuged at 11,500 g for 30 min. The mitochondrial pellet was resuspended in a KCl-Tris medium (120 mM KCl and 50 mM Tris-HCl, pH 7.4) and centrifuged at 12,500 g for 10 min. The final pellet was suspended in KCl-Tris medium, and protein concentration was determined by the method of Bradford described in Bio-Rad protein assay kit.

Animal care was in accordance with our university guidelines. Rats were maintained under a 12-h light and dark cycle in a temperature-regulated (23±1°C) animal room with water and food continuously available.

Measurement of membrane potential

The membrane potential change in mitochondria (1 mg of protein/ml) was assayed by measuring the fluorescence change of safranin O (Lee *et al.*, 2000). Assay mixture consists of mitochondria (1 mg of protein/ml), 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinate, 5 μM rotenone and 10 mM HEPES, pH 7.4. Fluorescence change was measured at an excitation wavelength of 495 nm and an emission wavelength of 586 nm using a luminescence spectrophotometer (Aminco-Bowman Series 2, Rochester, NY, USA).

Measurement of mitochondrial electron flow

The conversion of the dye MTT to formazan crystals in cells has been shown to be related to mitochondrial redox state and respiratory chain activity (Fu *et al.*, 1998; Cohen and Kesler, 1999). Mitochondrial fraction (0.2 mg of protein/ml) was suspended in the reaction mixture consisting of 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.2 and treated with 100 μM dopamine at 30°C for 15 min. The reaction was initiated by sequential addition of 0.11 mg/ml of MTT and pyruvate/malate (5 mM each), and absorbance was measured at 592 nm using a spectrophotometer (BECKMAN Instruments Inc., DU-70, Fullerton, CA, USA).

Measurement of cytochrome c release

Mitochondria were treated with dopamine and tyrosinase for the indicated time, and then the amount of cytochrome c in the supernatant, obtained by centrifugation, was determined. Mitochondria (1 mg of protein/ml) were incubated in a reaction mixture containing 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 3 μM rotenone, 0.8 μM antimycin A, 6 μM myxothiazol and 5 mM HEPES, pH 7.2, and treated with dopamine and tyrosinase for 15 min at 30°C. The reaction was

performed by the sequential addition of 0.1 mM potassium ferricyanide, 1 mM potassium cyanide and 0.1 mM sodium dithionite (Atlante *et al.*, 1999; Bustamante *et al.*, 2000). Absorbance change was measured at 550-540 nm using a dual wavelength spectrophotometer (Aminco-Chance, MD, USA). The amount of cytochrome c was determined using a molar extinction coefficient of $1.91\times10^4\ M^{-1}cm^{-1}$.

Measurement of thiol contents in mitochondria

Brain mitochondria (1 mg of protein/ml) suspended in 100 mM Tris-HCl buffer, pH 7.4 were treated with 100 μM dopamine and 50 units/ml tyrosinase for 30 min at 37°C. Thiol contents in mitochondria were determined using DTNB as described in the method of Lee *et al.* (2000).

Measurement of quinone and the melanin produced from dopamine

Dopamine quinone and melanin, the end product of dopamine oxidation, were measured as described in the previous reports (Offen *et al.*, 1996; Lai and Yu, 1997). The oxidation of dopamine by tyrosinase to produce dopamine quinone was measured at 479 nm. Reaction mixture was the same as described in the assay of melanin.

To assay melanin formation, mitochondria (1 mg of protein/ml) were incubated in the reaction mixture containing 5 mM dopamine, 120 mM KCl and 50 mM KH_2PO_4, pH 7.4. Absorbance of the supernatant obtained by centrifugation was measured at 405 nm, and the amount of melanin formed was determined using a commercial melanin (oxidation product of tyrosine) as the standard.

Statistical analysis

Data were expressed as means ± SEM values. Statistical analysis of the data was performed by one-way analysis of variance, which is followed by the Duncan's test. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of β-carbolines on the alteration of membrane potential and electron flow by dopamine oxidation

Dysfunction of brain mitochondria was measured with change in the membrane potential and electron flow. Brain mitochondria energized with succinate in the absence of Ca^{2+} showed a rapid fall of membrane potential, and then this potential maintained a level of steady state. Mitochondria pretreated with 100 μM dopamine (or plus 50 units/ml tyrosinase) for 15 min produced the membrane potential change consisting of a slow formation of polarization and subsequent depolarization. The similar finding is found in mitochondria pretreated with dopamine and iron (Kim *et al.*, 1999). This finding indicates that the oxidation products of dopamine cause alteration of the mitochondrial membrane potential. It has been shown that β-carbolines exert a neuroprotective effect against the toxicity of dopamine, glutamate and MPP^+ (Maher and Davis, 1996; Lee *et al.*, 2000; Kim *et al.*, 2001: Lee *et al.*, 2002b). Despite these reports, effect of β-carbolines on neurotoxicity of dopamine quinone has been uncertain. We examined the effect of β-carbolines on the membrane potential change due to dopamine oxidation. Harmaline (or harmalol, 100 μM each) and 10 μg/ml antioxidant enzymes (SOD and catalase) attenuated the dopamine-induced alteration of mitochondrial membrane potential (Fig. 1). In contrast to the effect of dopamine, 10 μg/ml antioxidant enzymes did not decrease the dopamine plus tyrosinase-induced alteration of membrane potential. Therefore, reactive oxygen species may not be involved in mitochondrial dysfunction caused by dopamine quinone. Harmalol (100 μM) attenuated the effect of dopamine plus tyrosinase, whereas harmaline did not show an inhibitory effect (Fig. 2). This finding indicates that β-carbolines may show a differential effect against toxic effect of dopamine oxidation products.

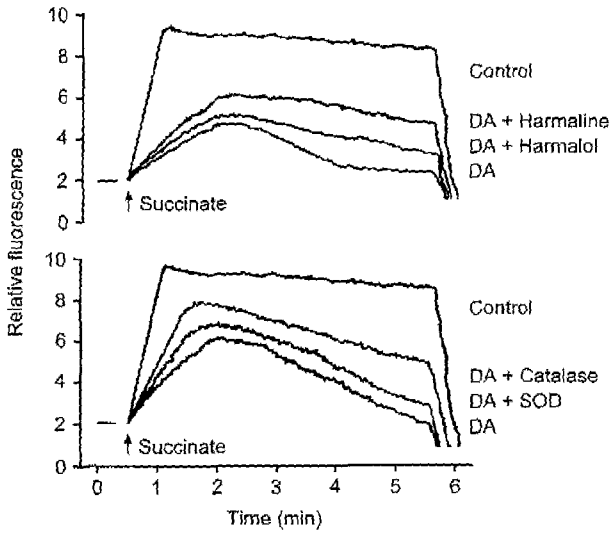

Fig. 1. Effect of harmaline and harmalol on dopamine oxidation-induced alteration of mitochondrial membrane potential. Brain mitochondria (1 mg of protein/ml) were treated with 100 μM dopamine (DA) in the presence or absence of compounds (100 μM β-carbolines, 10 μg/ml SOD and 10 μg/ml catalase) for 10 min, and then the formation of membrane potential was induced by the addition of 5 mM succinate. The traces are representative of 3-5 experiments.

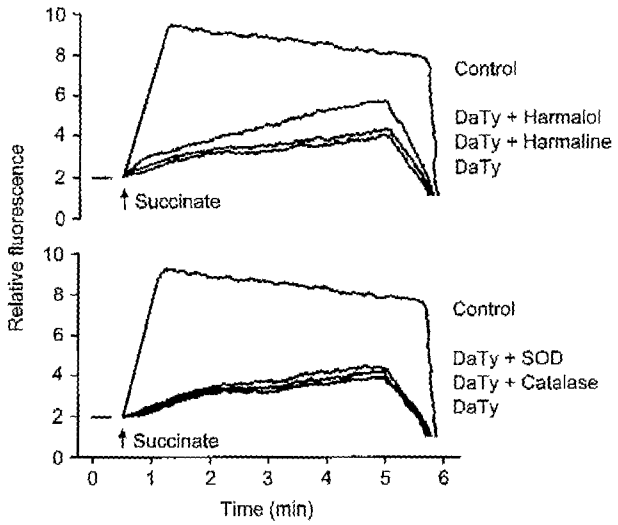

Fig. 2. Effect of harmaline and harmalol on the dopamine and tyrosinase-induced alteration of mitochondrial membrane potential. Brain mitochondria (1 mg of protein/ml) were treated with 100 μM dopamine plus 50 units/ml tyrosinase (DaTy) in the presence or absence of compounds (100 μM β-carbolines, 10 μg/ml SOD and 10 μg/ml catalase) for 15 min, and then the formation of membrane potential was induced by the addition of 5 mM succinate. The traces are representative of 3-5 experiments.

Inhibition of mitochondrial respiratory chain is suggested to play a role in the degeneration of nigrostriatal dopaminergic neurons. The 2-methyl form of β-carbolines at the concentrations from 185.7 to 840 μM are found to inhibit mitochondrial respiration energized with NAD^+ or succinate in the rat liver (Albores *et al.*, 1990). It has been shown that tyramine exerted a similar effect on both mitochondrial respiration and MTT reduction (Cohen and Kesler, 1999). Therefore, we examined the effect of indicated reagents on the mitochondrial respiratory chain with the electron flow. β-Carbolines (100 μM) and 100 μM dopamine (or plus tyrosinase) depressed the electron flow in mitochondria, respectively. However, the same concentrations of β-carbolines did not enhance the dopamine oxidation-induced inhibition of mitochondrial electron flow (Fig. 3). The results suggest that the effect of β-carbolines on the dopamine oxidation-induced mitochondrial damage may not be affected by their action on the respiratory chain.

Effect of β-carbolines on cytochrome c release and thiol oxidation

Opening of the membrane permeability pore has been hypothesized to induce the release of cytochrome c, which leads to apoptotic cell death (Chandra *et al.*, 2000). The effect of dopamine oxidation on the membrane permeability was examined its action on cytochrome c release from mitochon-

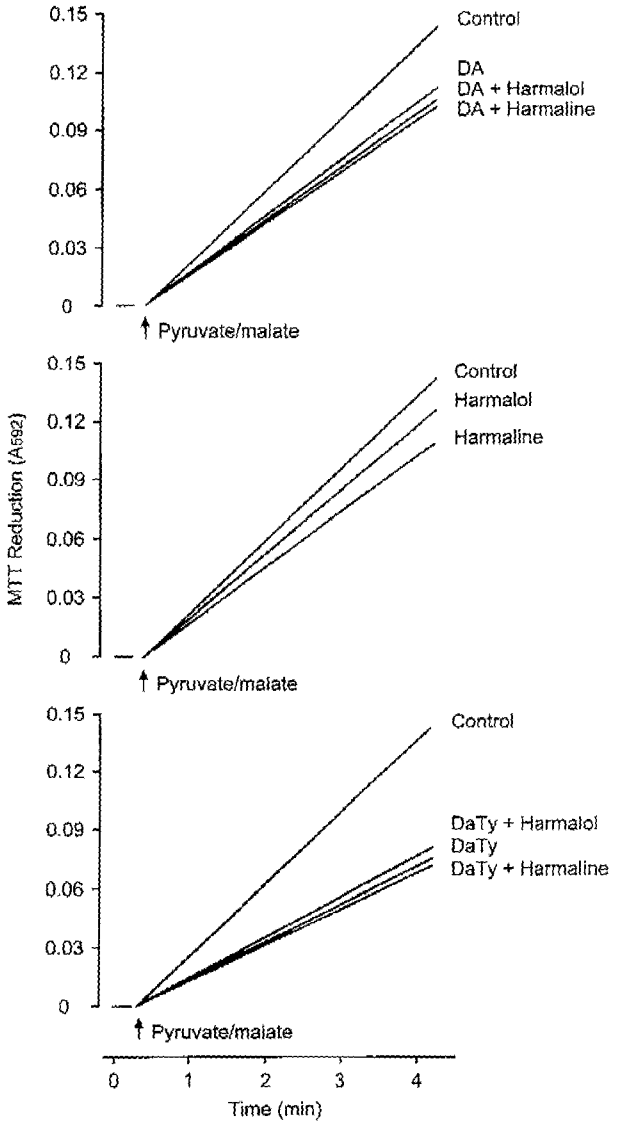

Fig. 3. Effect of β-carbolines on mitochondrial electron transport. Brain mitochondria (0.2 mg of protein/ml) were incubated with 100 μM of dopamine (DA or plus 50 units/ml tyrosinase (DaTy)) and 100 μM β-carbolines for 15 min at 30°C, and then the reaction was initiated by the addition of pyruvate and malate (5 mM each). Absorbance change due to MTT reduction was measured at 592 nm. The traces are representative of 3-5 experiments.

dria. Mitochondria treated with either 100 μM of dopamine or dopamine plus 50 units/ml tyrosinase for 15 min released 0.26 ±0.01 and 0.28±0.01 nmol cytochrome c/mg of protein, respectively (n=6). Antioxidant enzymes (10 μg/ml) attenuated the dopamine-induced release of cytochrome c, whereas they did not decrease the effect of dopamine plus tyrosinase (Fig. 4). Similar to change in the mitochondrial membrane potential, no effect of antioxidant enzymes suggests that dopamine quinone-induced cytochrome c release is not mediated by reactive oxy-

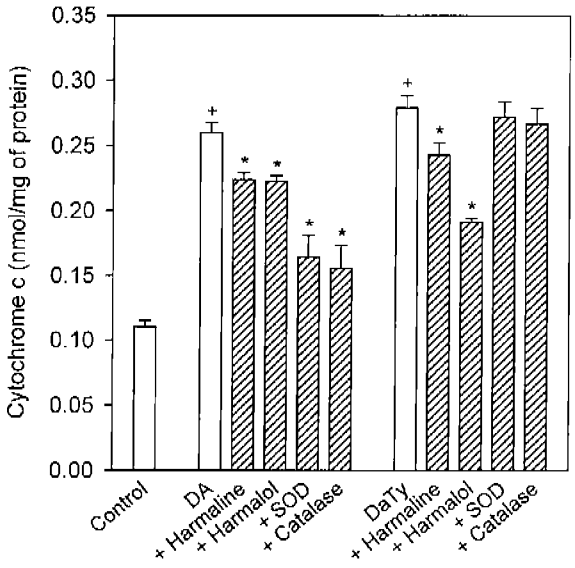

Fig. 4. Effect of β-carbolines on release of cytochrome c from mitochondria. Brain mitochondria (1 mg of protein/ml) were incubated with 100 μM of dopamine (DA or plus 50 units/ml tyrosinase (DaTy)), 100 μM β-carbolines, 10 μg/ml SOD or 10 μg/ml catalase for 15 min at 30°C. Data represent means ± SEM (n=6). *p<0.05, significantly different from dopamine alone or dopamine plus tyrosinase. +p<0.05, significantly different from the control.

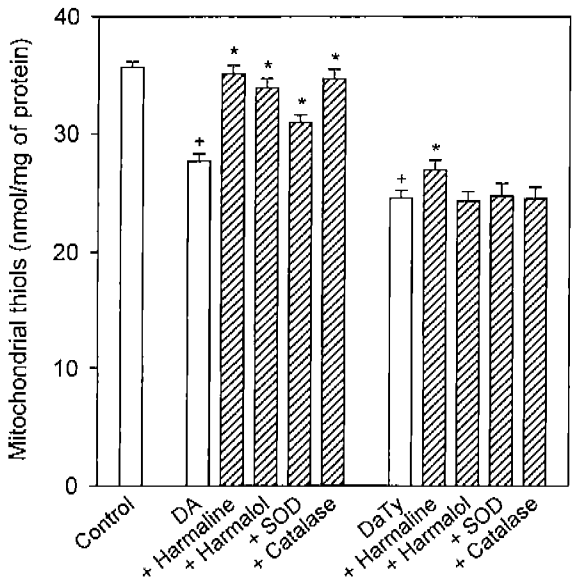

Fig. 5. Effect of β-carbolines on thiol oxidation in mitochondria. Brain mitochondria (1 mg of protein/ml) were incubated with 100 μM of dopamine (DA or plus 50 units/ml tyrosinase (DaTy)), 100 μM β-carbolines and 10 μg/ml of SOD (or catalase) for 30 min at 37°C. Data represent means ± SEM (n=5). *p<0.05, significantly different from dopamine alone or dopamine plus tyrosinase. +p<0.05, significantly different from the control.

gen species. Harmaline and harmalol (100 μM) reduced the effect of dopamine plus tyrosinase on cytochrome c release. The result indicates that β-carbolines may decrease change in the mitochondrial membrane permeability caused by the toxic action of dopamine plus tyrosinase.

The oxidant hydrogen peroxide induces the suppression of thiol-dependent respiratory chain (Cohen *et al.*, 1997). The membrane permeability of mitochondria may be modulated by the redox of dithiols and pyridine nucleotides (Reed and Savage, 1996; Rigobello *et al.*, 1998). The present study examined the effect of β-carbolines on the dopamine oxidation-induced thiol oxidation. The thiol content in intact mitochondria was 35.63±0.48 nmol/mg of protein. Either dopamine or dopamine plus tyrosinase caused 7.95 and 11.05 nmol/mg of protein of thiol oxidations in mitochondria, respectively. SOD and catalase (10 μg/ml) reduced dopamine-induced thiol oxidation in mitochondria, whereas they did not decrease the effect of dopamine plus tyrosinase. Harmaline (100 μM) inhibited a thiol oxidation in mitochondria caused either by dopamine or by dopamine plus tyrosinase (Fig. 5). Compared to harmaline, the harmalol did not significantly decrease the dopamine plus tyrosinase-induced thiol oxidation. The present finding indicates that the dopamine quinone-induced thiol oxidation may not be mediated by reactive oxygen species. It is unlikely that the oxidation of thiols alone plays a central role in cell death in neuronal cells (Olanow and Tatton, 1999). In this respect, protective effect of harmalol on the mitochondrial dysfunction induced by dopamine quinone may not be greatly affected by the effect on thiol oxidation.

Effect of β-carbolines on formation of melanin and quinone

The dopamine quinone and melanin has been shown to play a part in neuronal cell death in Parkinson's disease (Berman and Hastings, 1999; Stokes *et al.*, 1999). Effect of dopamine oxidation against brain mitochondria was determined by looking at the effect on the formation of melanin and quinone. Antioxidant enzymes (10 μg/ml) did not decrease the formation of dopamine quinone by tyrosinase. Incubation of mitochondria with 5 mM dopamine for 30 min produced a black color of melanin (47.81 μg/1 ml of mixture), which was significantly decreased by antioxidant enzymes. Harmaline and harmalol decreased the tyrosinase-induced formation of dopamine quinone and the formation of melanin from dopamine by mitochondrial MAO (Fig. 6). Inhibitory effect of antioxidant enzymes indicates that the reactive oxygen species produced from the dopamine oxidation by MAO may further increase oxidation of dopamine. Attenuation of iron and EDTA-mediated degradation of 2-deoxy-D-ribose by β-carbolines suggests

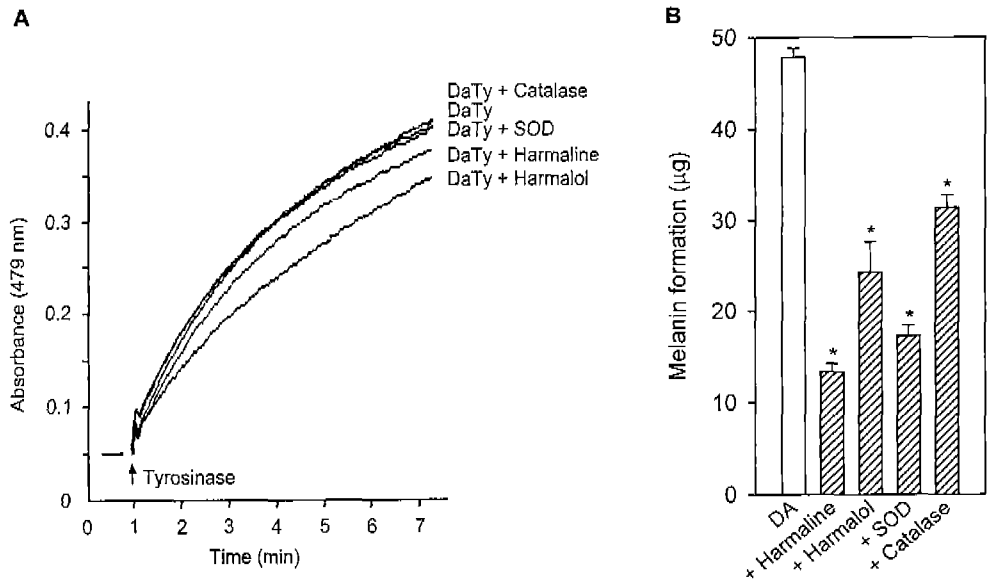

Fig. 6. Effect of β-carbolines on formation of dopamine quinone and melanin from dopamine. In dopamine quinone assay (A), dopamine (DA, 100 μM) was treated with 50 units/ml tyrosinase in the presence or absence of 100 μM β-carbolines and 10 μg/ml of SOD (or catalase) at 37°C. Absorbance change was measured at 479 nm. The traces are representative of 3-5 experiments. In melanin assay (B), brain mitochondria (1 mg of protein/ml) were incubated with 5 mM dopamine (DA), 100 μM β-carbolines, 10 μg/ml SOD or 10 μg/ml catalase for 30 min at 37°C. Data represent means ± SEM of micrograms (n=4). *$p<0.05$, significantly different from dopamine.

that the compounds have antioxidant effects (Lee *et al.*, 2000). Meanwhile, the toxic effect of the tyrosinase-induced oxidation of dopamine against mitochondrial function may not be accomplished by reactive oxygen species. Therefore, effect of β-carbolines on the mitochondrial dysfunction caused by dopamine plus tyrosinase was examined by looking at the effect on dopamine quinone and melanin. Compared to the MAO-induced dopamine oxidation, protective effect of β-carbolines against the brain mitochondrial dysfunction caused by the tyrosinase-induced oxidation of dopamine may be accomplished by decreasing formation of dopamine quinone and toxic substances other than reactive oxygen species.

In conclusion, β-carbolines may attenuate the dopamine quinone-induced brain mitochondrial dysfunction by decreasing the formation of reactive dopamine quinone and the change in membrane permeability. The elevated endogenous β-carbolines in the brains of patients with Parkinson's disease are suggested to exhibit the protective effect on neuronal cells against toxic action of dopamine quinone.

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