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Effect of ginsenoside Rb_1 on lipid peroxidation and neurotoxicity induced by MPTP in liver and brain of mouse

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Abbreviations: LPO, lipid peroxidation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; G-Rb_1, ginsenoside Rb_1; CL-HPLC, chemiluminescence-high performance liquid chromatography; PCOOH, phosphatidylcholine hydroperoxide; PKC, protein kinase C

Abstract

A neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to induce parkinsonism in rodents and human *via* dopaminergic cell death by a common oxidative mechanism. To find whether a neurotoxin-induced neurotoxicity is prevented by the treatment with an antioxidant, we investigated the effects of ginsenoside Rb_1, a major saponin from *Panax ginseng,* on lipid peroxidaton and neurotoxicity induced by MPTP in mice using chemiluminescense-HPLC. Levels of lipid hydroperoxides in plasma and liver were increased by MPTP treatment, but the increased levels of this were not observed in mice pretreated with ginsenoside Rb_1. Activities of protein kinase C and NADPH-cytochrome c reductase in the pretreated group with ginsenoside Rb_1 were lower than in MPTP groups. Treatment with ginsenoside Rb_1 was, however, less effective in suppressing the influence of MPTP on the levels of dopamine and its metabolite, homovanillic acid in the striatum of mice brain. These results indicate that ginsenoside Rb_1 has an antioxidant effect and may not have the ability enough to suppress the neurotoxicity induced by MPTP.

Keywords: lipid peroxidation, antioxidant, ginsenoside Rb_1, MPTP, dopamine, parkinsonism, chemiluminescense-HPLC

Introduction

Formation of oxygen radicals and lipid peroxidation (LPO) has been suggested to play a key role in various types of tissue degeneration and pathology such as aging, cancer, and retinal degeneration (Marx, 1987; Bulkley, 1983). Membrane phospholipids and triglycerides containing unsaturated fatty acids are thought to be the primary sites of the LPO process. The oxidation of membrane phospholipids has been hypothesized to cause an increase in the permeability of cell membranes and/or to inhibit membrane ion pumps and to lead to cell death. These oxdative stress is also related with the degeneration of dopaminergic cells.

Dopaminergic neurons are also destroyed during chronic exposure to amphetamines or after exposure to neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a contaminant of the illicitly synthesized meperidine (4-propionoxy-4-phenyl-1-methyl piperidine) (Gianni *et al.,* 1984) and is able to induce an irreversible Parkinson's disease-like syndrome in several animal species including primates (Langston *et al.,* 1983) and mice (Sonsalla, 1986). MPTP is metabolized by mitochondrial monoamine oxidase B to form 1-methyl-4-phenylpyridinium (MPP^+) (Markey *et al.,* 1984). Indeed, MPP^+ induces hydroxyl free radical formation when injected into the rat striatum (Wu *et al.,* 1993); moreover, the intracerebroventricular administration of MPP^+ increases striatal LPO in mice (Rolas *et al*., 1993). Some evidence from postmortem studies on Parkinson's disease (Bradbury *et al.,* 1986) suggested that excessive free radicals are involved in toxic effect on the dopaminergic neurons in the zona compacta of the subtantia nigra, and that the loss of protective mechanism against toxic free radicals may induce Parkinson's disease.

To overcome the effect of free radical and to reduce the neuronal damage by MPTP, the administration of antioxidants such as glutathione, ascorbic acid and vitamin E has been tried. Cleeter *et al*. (1992) demonstrated that the irreversible MPP^+-induced inhibition of mitochondrial complex I (Niklas *et al.,* 1985) is prevented by glutathione and ascorbic acid. The depletion of glutathione by MPTP in the brain stem of rat (Vizuete *et al.,* 1994) and in the subtantia nigra of mouse (Ferraro *et al.,* 1986) is prevented by pretreatment with the antioxidant α-tocopherol (Yong *et al.,* 1991). It has been reported that MPTP shows a greater neurotoxicity in vitamin E-deficient mice (Adams *et al.,* 1990).

Ginsenoside Rb_1 (G-Rb_1) (Figure 1) is a ginseng glycoside isolated from the root of *Panax ginseng.* G-Rb_1 has an antitumor effect (Motoo and Sawabu, 1994) as well as an antioxidant effect (Huang, 1989). The present study was undertaken to assess the effects of

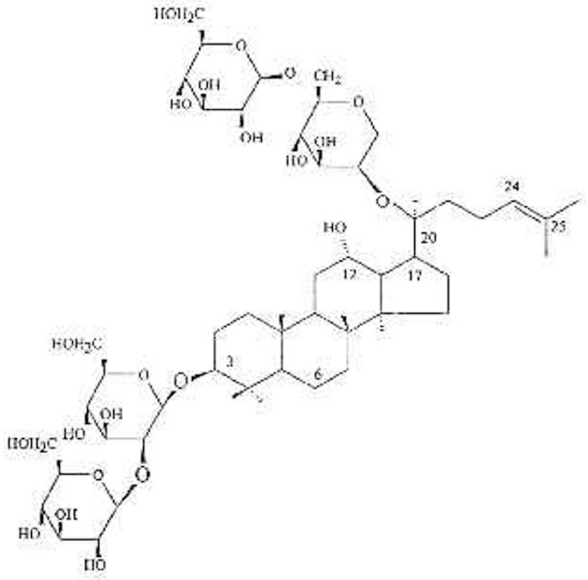

Figure 1. Structure of ginsenoside Rb_1

$G\text{-}Rb_1$ on lipid peroxidation and neurotoxicity induced by MPTP. In order to evaluate these effects, we measured the contents of phosphatidylcholine hydroperoxide (PCOOH) as a marker for LPO in the membrane of mice liver using chemiluminescence-high performance liquid chromatography (CL-HPLC) (Miyazawa *et al.,* 1987). The combination of normal-phase HPLC with chemiluminescence detector can serve as a sensitive and specific method for the improved quantitative assay of PCOOH *in vivo* and *in vitro*. To investigate the effect of $G\text{-}Rb_1$ and the correlation between lipid peroxidation and neurotoxicity induced by MPTP, we also measured the activities of protein kinase C (PKC) and NADPH-cytochrome c reductase in liver microsome of mouse, and the level of dopamine and homovanillic acid (the metabolite of dopamine) in the striatum of mouse brain.

Materials and Methods

Materials

MPTP was purchased from Aldrich (Milwaukee, U.S.A.). [γ-^{32}P]ATP and NADPH-cytochrome c reductase were obtained from BioRad (Hercules, U.S.A.). PKC assay kit was purchased from Amersham Life Science. $G\text{-}Rb_1$ (M.W. 1,108) donated from Korea Ginseng and Tobacco Research Institute (Taejon, Korea) was purified as described by Paik *et al.* (1982).

Animals and drug treatment

Male C57BL/6 mice (weighing about 25 g) were used in this study. The mice were divided into three groups; normal group (Normal), MPTP group (MPTP), and MPTP and $G\text{-}Rb_1$ treatment group (Rb_1+MPTP). Eight mice were housed per cage in a temperature (20 ± 2˚C) and light-controlled room (12/12 h, light/dark cycle) with free access to food and water. The $G\text{-}Rb_1$ were orally administrated at a dose of 10 mg/kg body weight once a day until the mice have been sacrificed. The MPTP was given subcutaneously at a dose of 10 mg/kg body weight once a day for 6 days, beginning at the 30th day of $G\text{-}Rb_1$ treatment. At the 10th day after final MPTP treatment, all animals including normal group were sacrificed by decapitation.

Preparation of blood plasma and liver tissue fractions

Plasma was isolated from the heparinized blood immediately after cardiac puncture. Liver tissues were homogenized in 2.0 ml of 0.32 M sucrose for 30 s using a Teflon-pestle homogeniser. Homogenate was centrifuged successively at 700, 9,000 and 105,000 *g* to obtain nuclear, mitochondrial, and microsomal fractions. The homogenate and tissue fractions were immediately frozen at -70˚C An aliquot of the 5% (w/v) homogenate was used for determination of protein concentration. The remainder was used for biochemical assay such as the content of lipid hydroperoxide and the activities of PKC and NADPH-cytochrome c reductase

Dissection of brain tissue

The brain was dissected to the striatum by the method of Dexter *et al*. (1989), frozen quickly with liquid nitrogen, and stored at -70˚C prior to dopamine analysis. Contents of dopamine and homovanillic acid in the striatum were assayed within a few days.

Determination of protein

The protein concentration in tissue samples was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Determination of PCOOH

Total lipid in plasma was extracted as described previously (Folch *et al*., 1957) (Figure 2). To determine the content of PCOOH in plasma and liver, 20 μl of each fraction was injected into CL-HPLC. The CL-HPLC system (Figure 3) consists of two parts, the separation of lipid by HPLC, and the postcolumn detection of hydroperoxide group by chemiluminescence reaction. The mobile phase A for the elution solvent was a mixture of hexane/isopropanol/methanol/water (5:7:2:1, v/v). The borate buffer B which

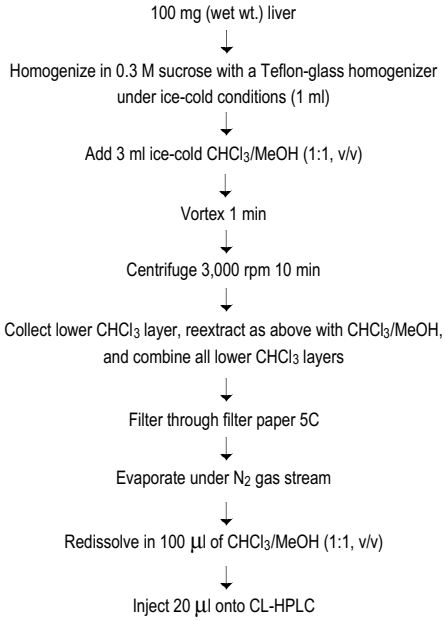

Figure 2. Proceeding diagram for extraction of lipid from liver tissue of mice for assay of phosphatidlycholine hydroperoxide by chemiluminescence-HPLC.

was a mixture of 10 mg/l cytochrome c and 2 μg/l luminol (Miyazawa *et al.* 1987) in 50 ml, was used as a chemiluminescence reagent. The flow rates of A and B were 1 ml/min, respectively. The concentration of PCOOH was estimated by calibration lines of authentic PCOOH, which was prepared by photoxidation of egg yolk phosphatidyl choline as described previously (1985). We used SIL-NH_2 column (4.6 mm×250 mm) (JASCO, Tokyo, Japan), and CLD-110 detector (Dohoku-Electric Company, Sendai, Japan).

The chemiluminescence reaction of lipid hydroperoxides is depicted in Figure 4. Lipid hydroperoxide (PCOOH) reacts with a mixture of cytochrome c and luminol dissolved in borate buffer B and yields a peroxyl radical (PCOO·). Singlet molecular oxygen ($^1O_2$) is formed together with lipid aldehyde (PCCHO) and alcohol (PCOH) by self reaction of two peroxyl radicals (Russell, 1957). The generated $^1O_2$ further oxidizes luminol and the oxyluminol emits the light of chemiluminescence at 432 nm.

Determination of activities of PKC and NADPH-cytochrome c reductase

The reaction mixture containing liver microsome (10

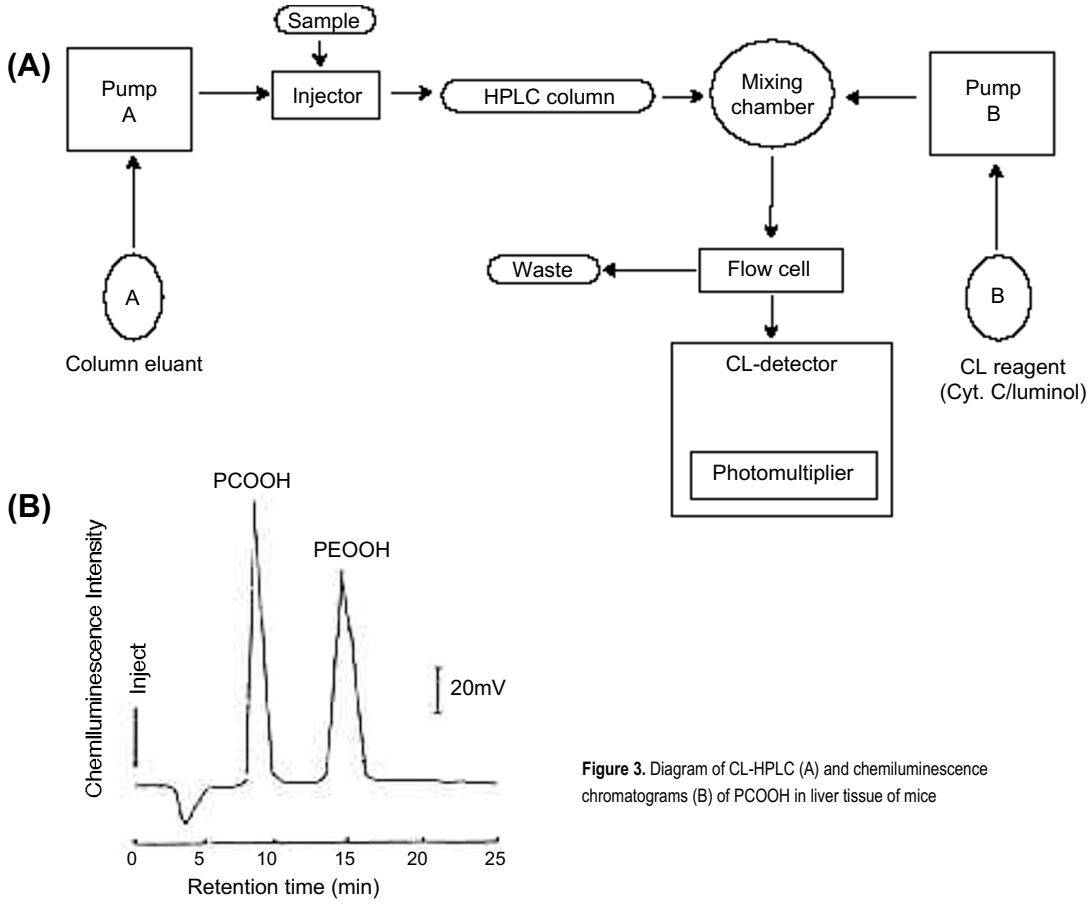

Figure 3. Diagram of CL-HPLC (A) and chemiluminescence chromatograms (B) of PCOOH in liver tissue of mice

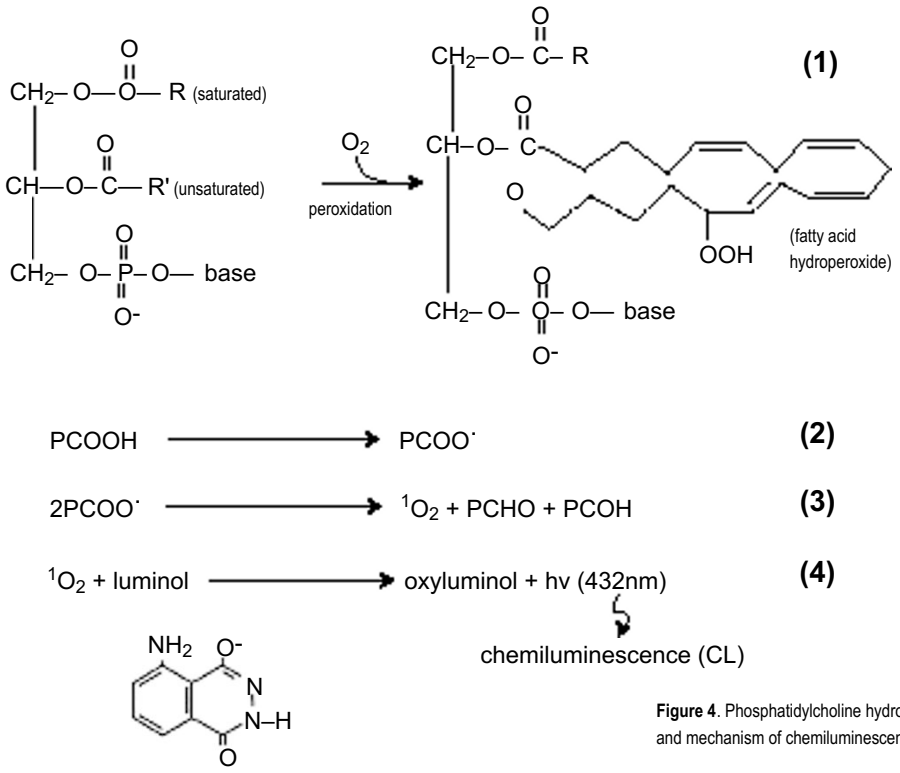

Figure 4. Phosphatidylcholine hydroperoxide (PCOOH) formation from phospholipid and mechanism of chemiluminescence reaction

μg/ml protein) and 2 μCi of [γ-^{32}P] ATP was incubated at 25˚C for 30 min. After PKC had been activated by phorbol-12-myristate-13-acetate, the activity of PKC was determined with an assay kit (Amersham Life Science). NADPH-cytochrome c reductase activity was determined with the following method (Bass *et al.,* 1967). The reaction mixture containing 0.5 M potassium phosphate buffer (pH 8.0), 0.2 mM cytochrome c and 0.5 mg liver homogenates was initiated by addition of 1 mM NADPH, and the change in the absorbance of this reaction mixture was measured during 3-5 min at 550 nm. The contents of reduced cytochrome c were determined from its extinction coefficient (21 $cm^{-1}mM^{-1}$ at 550 nm).

Determination of striatum dopamine and homovanillic acid

The striata of mouse brain were homogenized in 20 vol. of 0.1 M perchloric acid containing 0.1 mM sodium metasulfate, and the homogenates were centrifuged at 22,000 *g* for 15 min, and the supernatant was injected into the HPLC system. The contents of dopamine and homovanillic acid were measured simultaneously by reverse-phase HPLC (Lichspher 100 RP-18.5 μm, 250 ×4 mm) using eletrochemical detector (Esa, Coulochem II). The mobile phase was 0.15 M sodium phosphate (pH 3.4) containing 0.1 mM EDTA and 0.5 mM sodium octane sulfonic acid and the flow rate was 1 ml per min.

Stastics

Statistical analysis of the results was performed using Student's *t*-test.

Results

The effect of G-Rb1 on PCOOH formaton in mouse liver

To quantify LPO in membrane, it is necessary to measure the primary products such as PCOOH rather than to detect their metabolites. We measured PCOOH in mouse liver using CL-HPLC in this study. The PCOOH levels of MPTP group in cytosol, microsome, plasma, and mitochondria were 0, 32.8, 59.0 and 66.4nmol/mg lipid, respectively (Table 1). However, levels of PCOOH of Rb_1 + MPTP group in each subcellular fractions were negligible. These results indicate that the LPO was inhibited remarkably by the pretreatment with G-Rb_1

The activities of PKC and NADPH-cytochrome c reductase induced by MPTP

PKC, Ca^{2+}/phospholipid-dependent kinase, is known to be involved in the formation of oxygen free radicals by

Table 1. The effect of ginsenoside Rb_1 on PCOOH formation in liver tissue and plasma of mice.

Groups	PCOOH (nmol/mg lipid)[a]			
	Cytosol	Microsome	Mitochondria	Plasma
Normal	ND[b]	ND	ND	ND
MPTP	ND	32.8 ± 1.37	59.0 ± 3.83	66.4 ± 2.84
Rb_1+MPTP	ND	ND	< T[c]	< T

[a] All values are means ± standard deviation of five to six mice
[b] ND, none detected
[c] Below trace amount.

Table 2. The activities of PKC and NADPH cytochrome c reductase in liver microsome.

Groups	PKC (pmol/mg protein)	NADPH-cytochrome c reductase (nmol/mg protein)
Base[a]	0.32 ± 0.01	–
Normal	0.79 ± 0.13	36.4 ± 2.35
MPTP	1.51 ± 0.20	33.2 ± 1.50
Rb_1+MPTP	0.62 ± 0.03	21.9 ± 1.27

[a] Denotes basal level.

Table 3. The contents of dopamine and homovanillic acid (HVA) in striatum of mice brain.

Groups	Dopamine	HVA
	(μg/g tissue)	
Normal	7.86 ± 0.66	0.98 ± 0.01
MPTP	2.83 ± 0.50	0.52 ± 0.11
Rb_1+MPTP	2.84 ± 0.30	0.71 ± 0.04

stimulating the activity of NADPH-cytochrome c reductase (Kagan, 1988). Therefore, we measured the activities of both PKC and NADPH-cytochrome c reductase in the microsome of mice liver. The activity of PKC was measured after PKC had been activated by PMA (Muller, 1983). As shown in Table 2, the activity of PKC in MPTP group (1.5 pmol/mg protein) was two times higher than that of normal group (0.8 pmol/mg protein). However the activity of PKC in Rb_1+MPTP group (0.6 pmol/mg protein) was about 20% lower than that of normal group. The activity of NADPH-cytochrome c reductatse in the MPTP group showed higher activity (33.2 nmol/mg protein) than that (21.9 nmol/mg protein) of the Rb_1 + MPTP group. However, this activity of MPTP group was 10% lower than that (36.4 nmol/mg protein) of normal group. These results indicated that G-Rb_1 can reduce the activity of NADPH-cytochrome c reductase in normal and MPTP-treated groups.

Comparison of the contents of dopamine and homovanillic acid in striatum of mice brain

The dopamine content (2.83 μg/g tissue) in striatum of MPTP group was decreased to one-third of that (7.86 μg/g tissue) of normal group (Table 3). The dopamine level (2.84 μg/g tissue) of Rb_1 + MPTP group, however, did not show any significant difference from that of MPTP group. However, homovanillic acid content (0.71 μg/g tissue) of Rb_1 + MPTP group was higher than that (0.52 μg/g tissue) of MPTP group, corresponding to 70% and 50% of that (0.98 μg/g tissue) of normal group, respectively. From these results, it is suggested that the treatment of G-Rb_1 shows no suppressive activity in the oxidation of dopamine pathway by MPTP, but has a weak effect in inhibiting the degradation of homovanillic acid.

Discussion

The results of Table 1 show that the PCOOH formation in mitochondria of mouse liver treated with MPTP was much higher than that of any other groups. This result could be explained by the fact that monoamine oxidase B in the mitochondria converts MPTP to MPP^+ which plays an important role for its cytotoxic action (Heikkila *et al.,* 1984) as the ultimate toxic metabolite (Chiba *et al*., 1984), and that hydrogen peroxide and hydroxyl radicals are also produced by the interaction of MPP^+ with mitochondrial NADH dehydrogenase (Adams *et al.,* 1993). However, the PCOOH formation-induced by MPTP was prevented enough to be nondetectable in all groups by G-Rb_1 pretreatment. These results were consistent with the inhibitory effect of G-Rb_1 on ascorbate-NADPH induced-lipid peroxidation (Huang, 1989), and this was similar to the effect of vitamin E on AAPH and AMVN induced-PCOOH (Park, 1989).

Recently, a number of reports indicated that PKC may be activated following oxidative stress and that it may participate in LPO regulation to lead to cellular damage in biomembranes (Kagan *et al.,* 1988). The increment of PKC activity in MPTP group compared to that of normal group (Table 2) could be explained by the activation of PKC by increased levels of PCOOH in MPTP-treated group. It is also suggested that the activation of PKC by PCOOHs may be an early cellular response to oxidative stress induced by MPTP treatment. The decrease in the PKC activity of Rb_1+MPTP group also can be ascribed to the reduced oxdative stress with decreased formation of PCOOH by G-Rb_1 pretreatment.

NADPH-cytochrome c reductase is involved in the initiation and propagation of LPO in liver microsomes with 1:1:1 relationship between the amount of NADPH and O_2 consumed and the amount of H_2O_2 formed in the redox reaction. As shown in Table 2, the activity of NADPH-cytochrome c reductase in MPTP group was 10% lower than that of normal group. This result was consistent with the fact that the activity of NADPH-cytochrome c reductase could be inhibited by MPTP or MPP^+, because NADPH could not be oxidized by the conversion of MPTP to MPP^+ to promote the early formation of hydroxyl radicals to induce lipid peroxidation (Poirir *et al.,* 1985; Sinha *et al*., 1986). However, the reduction of cytochrome c in Rb_1 + MPTP group was 40% lower than that of MPTP group. This result might be due to the fact that NADPH-cytochrome c reductase was not activated when hydrogen peroxides induced by NADPH or MTPT had been scavenged by G-Rb_1 pretreatment.

It has been suggested that nonenzymatic and/or enzymatic oxidation of dopamine which was released by MPTP treatment may play a key role in the generation of $^{\cdot}OH$ free radicals in the iron-rich basal ganglia (Chueu et al 1994), in which Fe^+ ion in the pigmented dopamine-containing neurons catalyzes the autoxidation of dopamine and Fenton reaction (Iwahashi *et al.,* 1989) to produce cytotoxic $^{\cdot}OH$. This iron-induced damage of dopaminergic neurons in the substantia nigra might have resulted from a sequence of events including the $^{\cdot}OH$ generation and lipid perxidation (Mohanakumar *et al*., 1994). Thus, the $^{\cdot}OH$ free radical is the critical factor to lead to the degeneration of dopaminergic cell bodies in the substantia nigra (Javitch *et al.,* 1985) and a cause of nigra cell death in Parkinson's disease (Marsden, 1983). We found that the levels of dopamine and homovanillic acid in MPTP group were reduced to 30% and 50% of normal group, respectively (Table 3). These oxidations of dopamine and homovanillic acid in subtantia nigra of mice were consistent with the results of the production of toxic oxygen species such as superoxide and hydroperoxide (Perry *et al.,* 1982), and with Burn's report that the levels of dopamine and homovanillic acid in the striatum of monkey decreased to approximately 10% of control group when MPTP was administrated. Burn reported that the administration of MPTP was neurotoxic to nigrostriatal dopaminergic neuron in monkeys and caused Parkinson's disease (Burn *et al*., 1983). However, the level of homovanillic acid in Rb_1 + MPTP group was higher than that in MPTP group. Thes results indicate that G-Rb_1 showed a very limited effect in attenuating the depletion of dopamine induced by MPTP, and might have suppressed the metabolic process of dopamine. Therefore, the effect of G-Rb_1 might show a limited potency in this neuronal lesion, or it may be difficult for G-Rb_1 to penetrate into blood brain barrier.

In conclusion, the generation of free radical by MPTP caused the formation of lipid hyroperoxides in the liver. The correlation between the activities of PKC and NPDPH- cytochrome c reductase was inverse in mice treated with MPTP, and showed the decreasing trend in Rb_1 + MPTP group. We reached a conclusion that G-Rb_1 suppresses MPTP-induced lipid peroxidation with little inhibitory effect on MPTP-induced neurotoxicity. The results of this study encourage us to pursue further experiments in well defined system, since any hypotheses such as the report that the formation of toxic oxygen radicals by the administration of MPTP would cause disease (Longston *et al.,* 1983) have not been substantiated.

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