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Molecular mechanism of the relation of monoamine oxidase B and its inhibitors to Parkinson's disease: possible implications of glial cells

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Summary Monoamine oxidases A and B (MAO A and MAO B) are the major enzymes that catalyze the oxidative deamination of monoamine neurotaransmitters such as dopamine (DA), noradrenaline, and serotonin in the central and peripheral nervous systems. MAO B is mainly localized in glial cells. MAO B also oxidizes the xenobiotic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to a parkinsonism-producing neurotoxin, 1-methyl-4-phenyl-pyridinium (MPP+). MAO B may be closely related to the pathogenesis of Parkinson's disease (PD), in which neuromelanin-containing DA neurons in the substantia nigra projecting to the striatum in the brain selectively degenerate. MAO B degrades the neurotransmitter DA that is deficient in the nigro-striatal region in PD, and forms H_2O_2 and toxic aldehyde metabolites of DA. H_2O_2 produces highly toxic reactive oxygen species (ROS) by Fenton reaction that is catalyzed by iron and neuromelanin. MAO B inhibitors such as L-(−)-deprenyl (selegiline) and rasagiline are effective for the treatment of PD. Concerning the mechanism of the clinical efficacy of MAO B inhibitors in PD, the inhibition of DA degradation (a symptomatic effect) and also the prevention of the formation of neurotoxic DA metabolites, i.e., ROS and dopamine derived aldehydes have been speculated. As another mechanism of clinical efficacy, MAO B inhibitors such as selegiline are speculated to have neuroprotective effects to prevent progress of PD. The possible mechanism of neuroprotection of MAO B inhibitors may be related not only to MAO B inhibition but also to induction and activation of multiple factors for anti-oxidative stress and anti-apoptosis: i.e., catalase, superoxide dismutase 1 and 2, thioredoxin, Bcl-2, the cellular poly(ADP-ribosyl)ation, and binding to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Furthermore, it should be noted that selegiline increases production of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrphic factor (GDNF), possibly from glial cells, to protect neurons from inflammatory process.

Abbreviations: *BDNF* brain-derived neurotrophic factor, *CSF* cerebrospinal fluid, *DA* dopamine, *GDNF* glial cell line-derived neurotrophic factor, *MAO A* monoamine oxidase A, *MAO B* monoamine oxidase B, *MPDP+* 1-methyl-4-phenyl-2,3-dihydro-pyridinium, *MPP+* 1-methyl-4-phenyl-piridinium, *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *NGF* nerve growth factor, *PD* Parkinson's disease, *ROS* reactive oxygen species, *TH* tyrosine hydroxylase

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Parkinson's disease (PD) is an aging-related movement disorder caused by a deficiency of the neurotransmitter dopamine (DA) in the striatum of the brain as a result of selective degeneration of the nigro-striatal DA neurons (A9 neurons). DA deficiency is due to decreased number of DA neurons in the substantia nigra, and the molecular activity (enzyme activity/enzyme protein) of tyrosine 3-monooxygenase (tyrosine hydroxylase, TH) in residual DA neurons increases resulting in compensation for DA deficiency (Mogi et al., 1988). Familial PD for which the causative genes have been identified constitutes a small percentage of PD, and most PD is sporadic (idiopathic) without hereditary history. The molecular mechanism of the DA cell death in sporadic PD is unknown, but monoamine oxidase (MAO), especially type B (MAO B), is speculated to play important roles. In the brain MAO B is mainly localized in glial cells. MAO B activity in the brain increases during aging probably due to increasing numbers of glial cells (Fowler et al., 1980), and aging is a high risk factor of PD. MAO B inhibitors, such as L-(−)-deprenyl (selegiline) and rasagiline, are proved to be clinically effective for the treatment of PD. In the present review, we will examine the molecular mechanism of PD in relation to the mechanism of probable neuroprotection by MAO B inhibitors, and to possible interrelationship between DA neurons and glial cells in the inflammatory process.

Monoamine oxidases A and B (MAO A and MAO B)

Monoamine oxidase (flavin-containing) [amine:oxygen oxidoreductase (deaminating) (flavin-containing); MAO; E.C. 1.4.3.4.] catalyzes the following reaction: $RCH_2NH_2 + H_2O + O_2 = RCHO + NH_3 + H_2O_2$. MAO acts on primary

amines, and also on some secondary and tertiary amines. The monoamine substrates for MAO include physiologically and pathologically important neurotransmitters and hormones, such as DA, noradrenaline, adrenaline, and serotonin, which are slow-acting neurotransmitters in the brain and function with rapidly-acting neurotransmitters, i.e., glutamic acid and gamma-aminobutyric acid (GABA), to regulate movement, emotion, reward, cognition, memory, and learning. Thus, MAO is closely related to higher brain functions by regulating the levels of monoamine neurotransmitters and also to the pathogenesis of PD (for reviews, see Nagatsu, 2004; Nicotora et al., 2004; Riederer and Youdim, 1990; Youdim and Riederer, 1997). In the brain, MAO is thought to be important together with catechol O-methyltransferase in regulating the level of DA. The DA level decreases specifically in the nigro-striatal region in PD, which is the characteristic biochemical change (for reviews, see Cookson, 2005; Hornykiewicz, 2001; Nagatsu, 1993).

MAO was first discovered as tyramine oxidase by Hare in 1928, since it catalyzed the oxidative deamination of tyramine. This enzyme was then found to oxidize various monoamines including catecholamines, i.e., DA, noradrenaline, and adrenaline, and serotonine, and was recognized as monoamine oxidase by Blashko, Zeller, Gorkin, and Quastel. The enzyme localizes in the outer membrane of mitochondria (Schneitman et al., 1967). MAO was purified from bovine liver (Gomes et al., 1969; Minamiura and Yasunobu, 1978) and bovine brain (Harada et al., 1971). The cofactor, flavin adenine dinucleotide (FAD), was identified in preparations of purified MAO (Harada and Nagatsu, 1969; Tipton, 1980). Purified MAO was discovered to contain FAD covalently bound as 8-a-cysteinyl-FAD (Walker et al., 1971).

Johnston (1968) pharmacologically discovered that the inhibitor clorgyline was able to distinguish two forms of MAO, i.e., MAO type A (MAO A) and MAO type B (MAO B). The presence of multiple forms of MAO in the human brain was also reported by Collins et al. (1970). The structures and functions of MAO A and MAO B have been elucidated by cDNA cloning, genomic DNA cloning, and genetic engineering (for review, see Shih, 2004; Shih et al., 1999).

Full-length cDNAs encoding human liver MAO A and MAO B and the genomic DNAs were cloned (Bach et al., 1988; Chen et al., 1991; Powell et al., 1991; Weyler et al., 1990). Human placental MAO A (Hsu et al., 1988), and rat liver MAO A and MAO B (Ito et al., 1988; Kwan and Abell, 1992) were also cloned and sequenced, and human and rat MAO A showed 86–88% identity. MAO B from human platelets and frontal cortex were found to have identical amino acid sequences, confirming that human MAO B is a single enzyme in various tissues (Chen et al., 1993).

Human MAO A and MAO B have subunit molecular weights of 59,700 and 58,000, respectively, consisting of 527 and 520 amino acids, respectively, and have a 70% amino acid sequence identity; and both sequences contain the pentapeptide Ser-Gly-Gly-Cys-Tyr, in which the obligatory cofactor FAD is covalently bound through a thio ether linkage to the cysteine (Bach et al., 1988; Chen et al., 1991). MAO is composed of two identical subunits (Minamiura and Yasunobu, 1978), and one FAD couples with each subunit of 60 kDa (Weyler, 1989). FAD is covalently linked to Cys-406 in MAO A and Cys-397 in MAO-B (Abell and Kwan, 2001; Edmondson et al., 2004).

The expression of functional enzymes by transfection of cells with cDNAs provides unequivocal evidence that the different catalytic activities of MAO A and MAO B reside in their primary amino acid sequences. Chimeric enzymes and site-directed mutagenesis studies contributed to elucidating the structure-function relationships of MAO A and MAO B. The enzymatic properties observed for the chimeric MAO enzymes suggest that the internal segment, but not the N- or C-terminal region, confers substrate and inhibitor specificities (Shih et al., 1998; Tsugeno and Ito, 1997; Tsugeno et al., 1995). The catalytic properties and specificity of MAO A were insensitive to substitution of both the NH_2- (up to position 112) and COOH-termini (from residue 395). The replacement of MAO A amino acids 161–375 by the corresponding region of MAO B converted MAO A catalytic properties to ones typical of MAO B; and the converted enzyme did not oxidize serotonin, a preferred substrate of MAO A, and was more sensitive to the MAO B-specific inhibitor, L-(−)-derenyl (selegiline), than to the MAO A-specific inhibitor clorgyline. These results demonstrated that amino acids 152–366 of MAO B contain a domain that confers substrate specificity and inhibitor selectivity on the enzyme (Chen and Shih, 1998; Cesura et al., 1998).

Because MAO A and MAO B are integrated proteins of the outer membrane of mitochondria, their crystallization has been difficult; and so their three-dimensional structure of human MAO B has been only recently elucidated (Binda et al., 2002a, b). Determination of the crystal structure of human MAO B allowed precise modeling of the structure of human MAO A, and preliminary models of human MAO A have been obtained by fold recognition and comparative modeling based on proteins sharing low sequence identity (Leonard et al., 2004). The 50-residue C-terminal tail of human MAO B forms an extended segment that traverses the protein surface and then folds into an alpha-helix, which protrudes from the basal face of the structure to anchor the

protein to the mitochondrial outer membrane (Binda et al., 2002a, b).

MAO A and *MAO B* genes are situated on the X chromosome, at Xp 11.23–22.1 (Chen et al., 1992; Kochersperger et al., 1986; Lan et al., 1989; Levy et al., 1989; Pintar et al., 1981). Both genes are closely located and are deleted in patients with Norrie's disease, a rare X-linked recessive neurological disorder characterized by blindness, hearing loss, and mental retardation. Human *MAO A* and *MAO B* genes consist of 15 exons and have an identical exon-intron organization. Exon 12 codes for the covalent FAD-binding site and is the most conserved exon, showing 93.9% amino acid identity between MAO A and MAO B (Chen et al., 1992; Grimsby et al., 1991).

The distribution of MAO in various tissues of various species has been investigated by use of specific inhibitors of MAO A and MAO B enzyme activities, immunohistochemistry, enzyme autoradiography, and in situ hybridization (for review, see Berry et al., 1994; Kitahama et al., 1994; Luque et al., 1998). MAO A and MAO B are distributed in various tissues including the brain of various species. Histochemical localization of MAO A and MAO B was examined in the rat brain (Willonghby et al., 1988). In the rat brain, MAO A was predominantly found in noradrenergic neurons; whereas MAO B was detected in serotonergic and histaminergic neurons and in glial cells (astrocytes) (Arai et al., 1997; Jahung et al., 1997; Levitt et al., 1982; Luque et al., 1995; Saura et al., 1994; Westlund et al., 1988a). However, DA neurons appear not to have MAO A or MAO B, in contrast to the fact that DA is a common substrate of both MAO A and MAO B activity (Arai et al., 1998; Hida et al., 1999). As another puzzling fact on the physiological role of MAO B, serotonin neurons contain only MAO B, but serotonin is a very poor substrate of MAO B (Arai et al., 1997; Levitt et al., 1982).

Most human tissues, including the brain, express both MAO A and MAO B (Konradi et al., 1988; Konradi et al., 1989; Westlund et al., 1988b). However, human placenta contains predominantly MAO A (Egashira and Yamanaka, 1981); and human platelets and lymphocytes express only MAO B (Bond and Cundall, 1997; Donnelly and Murphy, 1977). Thus platelet MAO B can be useful for estimation of brain MAO B (Oreland, 2004). mRNA transcripts of MAO A and MAO B were coexpressed in the same region in the adult human brain; and the relative concentrations of these transcripts were as follows in the decreasing order: frontal cortex, locus coeruleus, temporal cortex, posterior pensylvian cortex-supuramarginal gyri, anterior pensylvian cortex-opercular gyri, hippocampus and thalamus (Grimsby et al., 1990).

Cell-type specific expression of MAO A and MAO B were examined in cultured cells (Donnelly et al., 1976; Hawkins and Breakfield, 1978; Murphy et al., 1976; Nagatsu et al., 1981). The type of MAO activity did not vary through the stage of growth of mouse myoblast G8-1 cells, which contain mostly MAO A (95%) and a small amount of MAO B (5%) (Nagatsu et al., 1981). NG108-15 hybrid cells derived from mouse neuroblastoma × rat glioma showed both MAO A (65–90%) and MAO B (35–10%), and the total MAO A plus MAO B activity and the ratio of MAO B/MAO A activity increased as a function of time in culture. Prostaglandin E1 and theophylline, the best known combination of agents that increases the intracellular cyclic AMP content of NG-108-15 cells, caused similar increases in MAO and the MAO B/MAO A ratio in NG108-15 cells, suggesting that the activity and expression of MAO B are regulated in a cyclic AMP-dependent manner (Nakano et al., 1985). NCB 20 cells, which are a hybrid of mouse neuroblastoma N18TG-2 and Chinese hamster embryonic brain cells CHB C, had predominantly MAO B activity with a little MAO A activity (Nagatsu et al., 1981). MAO B and MAO A in hybrid NCB 20 cells were determined to be distinct enzyme molecules by peptide mapping (Nakano et al., 1986).

MAO B activity, but not MAO A activity in the brain increases during aging (Fowler et al., 1980). This increase may be due to the increase in the number of glial cells during aging. In the living human brain, MAO B can be detected by positron emission tomography (PET) using deuterium substituted [^{11}C] L-(−)-deprenyl (selegiline) (Fowler et al., 1998). The PET study indicated that MAO levels in the human brain were highest in the basal ganglia and the thalamus, intermediate in the frontal cortex and cingulate gyrus, and lowest in the parietal and temporal cortices and cerebellum. The results of PET confirm postmortem studies on increases in brain MAO B with age. The whole brain and the cortical regions and the basal ganglia, thalamus, pons, and cerebellum showed an average increase of 7.1 ± 1.3% per decade. There was also a large variability among subjects in the same age range. Interestingly, inhibition of MAO B was observed by PET study in the brain of smokers (Fowler et al., 1996). Smokers also showed low MAO B in platerets (Olerand, 2004), and are speculated to have a low incidence of PD.

MPTP-induced Parkinsonism and monoamine oxidase B (MAO B)

The discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as the first recognized synthetic neurotoxin

that is capable of inducing PD symptoms in humans has greatly contributed to the understanding of the molecular mechanism of sporadic PD. Calne and Langston (1983) reviewed the etiology of PD and pointed out the possibility of involvement of environmental toxic substances as being the important cause of PD, superimposed on a background of slow, sustained neuronal loss due to the process of aging. Humans are highly susceptible to MPTP, and non-human primates are also sensitive to the compound. Various non-primate animals including some strains of the mouse and even the fruit fly Drosophila also show PD-like movement disorder by administration of MPTP. The first human case of PD that appeared after intravenous injection of MPTP as a contaminant of 1-methyl-4-phenyl-piperidine-4-carboxulic acid ethyl ester (meperidine), which is a synthetic heroin, was a 23-year-old chemistry student at Bethesda, MD, USA. He synthesized that meperidine containing MPTP as a by-product and injected it intravenously into himself. L-3,4-Dihydroxyphenylalanine (L-DOPA) to supplement DA in the brain was effective in that patient as in PD patients. Kopin's group at the National Institutes of Health (NIH) identified MPTP in that meperidine preparation and reported the case in 1979 (Davis et al., 1979). Then, in 1983 in California, a group of young drug addicts acutely showed PD-like symptoms after self-administration of street batches of meperidine contaminated by MPTP. Like idiopathic PD, L-DOPA, which supplements DA in the brain as a substrate of aromatic L-amino acid decarboxylase, was an effective cure for the symptoms. These cases were reported by Langston et al. (1983), and since then the molecular mechanism of MPTP-elicited PD and investigation of similar neurotoxins in environment have been extensively studied (for review, see Nagatsu, 1997, 2002b). MPTP is highly lipophilic, and after its systemic injection, it rapidly crosses the blood-brain barrier to enter the brain. Once in the brain, MPTP, which is a pro-neurotoxin, is metabolized to 1-methyl-4-pheny-2,3-dihydro-pyridinium (MPDP+), by MAO B, which is localized in the outer membrane of mitochondria within glial cells. MPDP+ is then probably spontaneously oxidized to 1-methyl-4-phenyl-pyridinium (MPP+), the active PD-producing neurotoxin. MPP+ is then taken up via DA-transporter across the plasma membrane at the nerve terminals of the nigro-striatal DA neurons in the striatum. As acute reactions, MPP+ is taken up into synaptic vesicles from the cytoplasm by vesicular monoamine transporter type 2 (VMAT 2) to release DA from the nerve terminals; it also inhibits and inactivates tyrosine hydroxylase (TH) to decrease DA synthesis. In the chronic phase, MPP+ is transported from the nerve terminals of nigro-striatal DA neurons in the striatum to the cell bodies in the substantia nigra by retrograde axonal flow. MPP+ is also accumulated within the inner mitochondrial membrane, where it inhibits complex I (NADH ubiquinone oxidoreductase), one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation for ATP fromation, interrupts electron transport, releases reactive oxygen species (ROS) causing oxidative stress, and depletes ATP. Inhibition of mitochondrial complex I opens mitochondrial permeability transition pore, and subsequently triggers apoptotic cell death of the nigro-striatal DA neurons. Thus, MPP+ decreases DA acutely and chronically to produce PD-like symptoms. Oxidation of MPTP to MPP+ by mitochondrial MAO B in glial cells is essential for neurotoxicity, and selegiline as a specific MAO B inhibitor completely prevents the symptom of PD by MPTP. Mitochondrial dysfunction, especially decreased activity of complex I, is confirmed in the nigro-striatal region in the brain in sporadic PD (for review, see Mizuno et al., 1998). However, unlike sporadic PD, Lewy bodies are not observed in the remaining neurons in the substantia nigra in MPTP-induced PD.

Assuming that some MPTP-like neurotoxins in environment may trigger idiopathic PD, endogenous MPTP-like compounds have been investigated in postmortem brains and in the cerebrospinal fluid (CSF) from patients with PD. Two groups of MPTP-like compounds, isoquinolines (IQs) and beta-carbolines, were found in the human brains and CSF from patients with PD.

We found that MPP+ acutely inhibits the TH system in tissue slices of the rat striatum. In screening for various MPTP-like compounds that inhibit the striatal TH system, we found tetrahydroisoquinoline (TIQ) and its derivatives to be active inhibitors (Hirata et al., 1986). Tetrahydroisoquinoline alkaloids were first discovered in the brain as an in vivo metabolite of L-DOPA in humans by Sandler et al. (1973). Various TIQs were found in the brains of patients with PD and in those of non-parkinsonian control patients by gas chromatography/mass spectrometry: TIQ, 1-methyl-TIQ (1-Me-TIQ), N-Me-6,7-$(OH)_2$-TIQ, (N-Me-norsalsolinol), 1,N-$(Me)_2$-6,7-$(OH)_2$-TIQ (N-Me-salsolinol), 1-phenyl-TIQ, N-Me-1-phenyl-TIQ, and 1-benzyl-TIQ (1-Bn-TIQ) (for review, see Nagatsu, 1997, 2002b; Niwa et al., 1993). Exogenously administered TIQ easily crosses the blood-brain barrier and passes into the brain. However, endogenous TIQs in the brain are speculated to be enzymatically synthesized from precursor endogenous monoamines such as phenylethylamine or DA. Only the (R) enantiomer, (R)-N-Me-6,7-$(OH)_2$-TIQ (R-N-Me-salsolinol) is speculated to be enzymatically synthesized in the brain (Naoi et al., 1996). Among these TIQs in the brain, 1,N-$(Me)_2$-6,7-$(OH)_2$-TIQ (N-Me-salsolinol)

(Naoi et al., 1996), N-Me-6,7-$(OH)_2$-TIQ (N-Me-norsalsolinol) (Moser and Koempf, 1992), and 1-Bn-TIQ (Kotake et al., 1995, 1998) have been extensively investigated as probable neurotoxins to cause PD. It was also suggested that some cases of atypical PD in the French West Indies might have a link with the consumption as food of tropical plants that contain Bn-TIQs (Caparros-Lefebvre et al., 1999). Beta-carbolines have structures similar to those of MPTP/MPP+, and may be synthesized in vivo from tryptophan via tryptamine (Collins and Neafsey, 2000; Matsubara, 2000). A neurotoxic 2,9-dimethylated beta-carbonium, 2,9-dimethylated norharman, was found by gas chromatography/mass spectromrtry in CSF in half of the PD patients examined, but was not found in non-PD patients. 1-Trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) is another neurotoxic beta-carboline (Bringmann et al., 2000). TaClo can be synthesized in vivo from tryptamine and the synthetic chloral after application of the hypnotic chloral hydrate or after exposure to the widely used industrial solvent trichloroethylene, which is metabolized to chloral (Bringmann et al., 2000). However, since TaClo and the N-methylated derivative had no DA-transporter-mediated neurotoxicity in cultured cells transfected with the human DA-transporter gene, they may not cause neurotoxicity by a mechanism analogous to that of MPTP/MPP+ involving the uptake into DA neurons by DA-transporter.

Like MPTP, the neurotoxicity of 1-Bn-TIQ (Kotake et al., 1998), N-methyl-(R)-salsolinol (Naoi et al., 1996), and beta-carbolines (Collins and Neafsey, 2000; Matsubara, 2000) are suggested to be precursor neurotoxins, and to be protected by MAO B inhibitors. These compounds inhibit complex I to reduce ATP synthesis in agreement with low complex I activity in the brain in PD and may produce ROS.

Rotenone is a naturally occurring, lipophilic compound from the roots of certain plants (Derris species) with the structure not related to amines, and is used as the main component of many insecticides. Rotenone is a specific inhibitor of complex I, and in Lewis rats by the chronic systemic administration causes highly selective degeneration of the nigro-striatal DA neurons with behavioral PD symptoms of hypokinesia and rigidity and with formation of intracytoplasmic inclusions like Lewy bodies, which are mainly composed of alpha-synuclein and a characteristic feature of sporadic PD (Betarbet et al., 2000). The relation of rotenone to MAO B remains to be investigated.

Specific inhibitory activity towards complex I of IQs and beta-carbolines suggests that they might be the possible neurotoxins producing PD. However, the concentrations of IQs and beta-carbolines in postmortem brain and CSF are low (in the order of ng/g tissue), and their in vivo toxicity and clinical significance in human PD remain to be further examined. Also, the question remains; is there any relation between clinical efficacy of MAO B inhibitor L-deprenyl (selegiline) in PD patients, as describes below, and complete prevention of PD symptoms in animal PD-models produced by MPTP- or MPTP-like neurotoxins by the inhibitor?

Clinical efficacy of monoamine oxidase B inhibitors in Parkinson's disease

L-Deprenyl (R-(−)-deprenyl, the generic name selegiline) was the first discovered MAO B specific inhibitor (for review, see Knoll and Magyar, 1972; Knoll, 1980). Selegiline is a suicide inhibitor, i.e., the compound acts as a substrate for the target enzyme MAO B and results in irreversible inhibition (Riederer and Youdim, 1990). Clinical efficacy of the MAO B inhibitor, selegiline, for addition of L-DOPA that supplements deficient DA in PD was first reported by Birkmayer et al. (1985). In a long term (9 years) study of treatment of PD patients with L-DOPA alone or in combination with selegiline, a significant increase of life expectancy in L-DOPA-selegiline group was observed. The results were interpreted as indicating selegiline's ability to prevent or retard the degeneration of striatal DA neurons. This hypothesis was not far fetched since selegiline selectively prevents the degeneration of nigro-striatal DA neurons in animal PD models induced by MPTP, as described above. After the first work on the clinical efficacy of selegiline on Parkinson's disease (Birkmeyer et al., 1985), the Parkinson Study Group in USA (1989) preliminarily reported that the use of selegiline (10 mg per day) delays the onset of disability associated with early, other untreated cases of PD. The Parkinson Study Group (1993) further reported the results of the multicenter controlled clinical trial of Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (the "DATATOP" study). Selegiline and tocopherol (vitamin E as an antioxidant) clinical trial from 1987 for 5 years (the US DATATOP study, selegiline momotherapy) suggested that deprenyl (10 mg per day) but not tocopherol (2000 IU per day) dalays the onset of disability associated with early, otherwise untreated PD. However, this remains controversial (Lang and Lees, 2002). Further uncertainty arose in 1995, when a study by the Parkinson's Disease Research Group of the United Kingdom (UK-PDRG) found 57% higher mortality in patients receiving combined selegiline and L-DOPA treatment compared with patients on L-DOPA alone (Lees on behalf of the Parkinson's Disease Research

Group of the United Kingdom, 1995). Other clinical trials have, however, failed to show any increase in mortality and showed neuroprotective effects of selegiline (Counsell, 1998; Olanow and Riederer, 1996; Olanow et al., 1995). Furthermore, another MAO B inhibitor, rasagiline (N-propargyl-R-aminoindan) that is a selsective, irreversible, second-generation MAO B inhibitor, has shown effectiveness in early PD when given as once-daily treatment without dose titration (Parkinson Study Group, 2002). To clarify the role of MAO B inhibitors in the treatment of PD, Ives et al. (2004) did a meta-analysis of data from all published trials, and reported that MAO B inhibitors (selegiline, lazabemide, or rasagiline) with or without L-DOPA, versus placebo, L-DOPA, or both, reduce the need for L-DOPA, and the incidence of motor fluctuations, without substantial side effects or increased mortality. This study supported the efficacy and safety of monotherapy of early PD by MAO B inhibitors such as selegiline.

Molecular mechanism of neuroprotective effects of L-deprenyl (selegiline) against Parkinson's disease

Stimulated by clinical efficacy of selegiline as a MAO B inhibitor for the treatment of early PD as described above, mechanisms of possible neuroprotection by selegiline have been extensively studied. The early hypothesis on the mechanism of clinical efficacy of selegiline in the treatment without or with L-DOPA was the prevention of degradation of DA, which is produced endogenously from tyrosine by TH or from exogenously administered L-DOPA for treatment, by MAO B inhibition (symptomatic effect). However, accumulating results indicate that selegiline may also have neuroprotective effects by several mechanisms that are related or not related to MAO B inhibition.

Neuroprotection due to inhibition of dopamine degradation by MAO B inhibitor selegiline

DA is a common substrate of MAO B and MAO A. However, in PD only MAO B inhibitor is clinically effective. Selegiline may increase the level of DA in the synaptic cleft in the DA nerve endings in the striatum after release from presynaptic terminals by inhibiting MAO B. DA as a substrate of MAO B produces H_2O_2 and 3,4-dihydroxyphenylacetaldehyde as neurotoxic products. However, since presence of MAO activity is not observed in DA neurons (Arai et al., 1998), DA released from DA neurons or produced from exogenously administered L-DOPA in L-DOPA therapy may be oxidized in the outside of DA neurons possibly in glial cells that contain MAO B to produce cytotoxic H_2O_2 and the aldehyde metabolite. Then H_2O_2 may get into the nigro-striatal DA nerons, and may be oxidized to produce cytotoxic oxygen radicals (reactive oxygen species, ROS) by iron presumably catalytically with neuromelanine. Iron accumulates in the DA neurons in the substantia nigra in PD (Dexter et al., 1987; Hirsch et al., 1991; Jellinger et al., 1992; Sofic et al., 1988). ROS may cause lipid membrane peroxidation and finally cell death of DA neurons (Dexter et al., 1993; Youdim et al., 1993). MAO B inhibitors can prevent this neurotoxic process to protect DA neurons.

Another possible mechanism of selegiline related to MAO B inhibition is an amphetamine-like tonic effect due to increased accumulation of phenylethylamine. Phenylethylamine is a good substrate of MAO B and may be produced in glial cells. Phenylethylamine at high concentrations were found in the striatum in the postmortem brain from PD patients treated with selegiline, and may have an endogenous "amphetamine-like activity" to stimulate DA neurons (Gerlach et al., 1992).

Selegiline's neuroprotective mechanism that is not related to MAO B inhibition

It has been known for many years that neuroprotective effects of selegiline can be observed in cell culture experiments at lower concentrations than those for MAO B inhibition, suggesting that selegiline's neuroprotective effects may also be caused by some other mechanisms than MAO B inhibition.

Riederer and Lachenmayer (2003) pointed out the possibility of neuroprotection by selegiline independent from MAO B inhibition by re-examining the clinical studies such as the DATATOP study (1993) based on the half life of selegiline in vivo in humans. In those clinical studies, the efficacy of selegiline was evaluated at the end-point between baseline and the end of the study (14 months including a 2 months wash-out period). Reported data on the half life of seligiline were between about 2–10 days (Gerlach et al., 2003; Youdim and Tipton, 2002) and 40 days (Fowler et al., 1994). Even the slow recovery of MAO B activity as determined by Fowler et al. (1994) would indicate only a 20% recovery of MAO B activity after a 2-week wash-out period and less than 50% recovery after a 4-week period. However, a significant increase in amine neurotransmitter concentrations can only be demonstrated after the MAO activity has been inhibited by at least 80% (Green et al., 1977). Thus a recovery of only 20% of the MAO B activity is already sufficient to prevent an increase in the neurotransmitter concentration. These results would suggest that

a safe period of 4 weeks for wash-out of selegiline would be perfectly adequate for avoidance of any residual symptomatic effects by in vivo MAO B inhibition and that the residual efficacy indicates its neuroprotective effectiveness of this class of drugs as a cornerstone of drug development not only for PD but also for neurodegenerative disorders in general (Riederer and Lachenmayer, 2003). In experimental animals selegiline was shown to be protective against the damaging effects of several neurotoxins, including the dopaminergic neurotoxin MPTP and 6-hydroxydopamine (6-OHDA) and the noradrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), again suggesting that selegiline may show neuroprotective mechanism of action which is independent of its action on MAO B (Gerlach et al., 1992). Furthermore, selegiline dose-dependently attenuated ethylcholine aziridinium ion-induced memory impairment, and co-administration of selegiline and donepezil, a selective acetylcholinesterase inhibitor, at doses that do not exert efficacy individually, significantly ameliorated scopolamine + p-chlorophenylalanine-induced memory deficits (Takahata et al., 2005a).

There have been several suggestive findings on the molecular mechanism of neuroprotection by MAO B inhibitor selegiline.

First, selegiline and the metabolite desmethylselsegiline stimulated synthesis of neurotrophins, i.e., nerve rrowth factor (NGF), brain- derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF), which act for neuroprotection and anti-apoptosis, in cultured mouse astrocytes (Mizuta et al., 2000). Selegiline as well as BDNF showed trophic effects on cultured DA neurons (Kontkanen and Castren, 1999). Besides neuroprotection for DA neurons, in mixed primary cultures of hippocampal neuronal and glial cells, selegiline increased NGF protein content and protected hippocampal neurons from excitotoxic degeneration, suggesting that astrocyte-derived NGF could contribute to the neuroprotective activity (Semkova et al., 1996).

Second, selegiline increased the activity of catalase and Mn-superoxide dismutase (Mn-SOD; SOD 2) in the striatum of 25-week-old rats. In slice cultures, selegiline increased Cu, Zn-superoxide dismutase (Cu, Zn-SOD; SOD 1) and Mn-SOD activities with a maximal effective concentration of 10^{-8} and 10^{-10} M, respectively. Furthermore, selegiline significantly increased glutathione level (Takahata et al., 2005b). Selegiline, at 1 μM or less, induced thioredoxin for protection against oxidative injury caused by MPP + in human SH-SY5Y neroblastoma cells and also in primary neuronal culture of mouse midbrain DA neurons. The redox cycling of thioredoxin may mediate the protective action of selegiline. Thioredoxin at 1 μM increased the expression of mitochondrial proteins Mn-SOD and Bcl-2 supporting cell survival (Andoh et al., 2002). Thus selegiline without modifying MAO B activity may augment the gene induction of thioredoxin leading to elevated expression of anti-oxidative Mn-SOD and anti-apoptotic Bcl-2 protein in the mitochondria for protecting against MPP +-induced neurotoxicity. The induction of thioredoxin was blocked by a protein kinase A (PKA) inhibitor and mediated by a PKA-sensitive phospho-activation of MAP kinase ERK 1/2 and transcription factor c-Myc. Selegiline-induced thioredoxin and associated neuroprotection were concomitantly blocked by the antisense against thioredoxin mRNA (Andoh et al., 2005). These results suggest that selegiline can decrease oxidative stress in the nigro-striatal region by augmenting various anti-oxidant systems.

Third, selegiline was found to alter the cellular poly(ADP-ribosyl)ation response to gamma-irradiation. Because poly(ADP-ribose) formation is catalyzed by the 113-kDa nuclear enzyme poly(ADP-ribose)polymerase 1 (PARP-1), this result suggests that altered cellular PARP-1 activity may contribute to the neuroprotective potential and/or life span extention afforded by selegiline (Brabeck et al., 2003).

Fourth, selegiline and other propargylamines were found to bind to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The GAPDH binding was associated with decreased synthesis of pro-apoptotic protein, and thus may contribute to neuroprotection (Tatton et al., 2003).

All these results suggest anti-oxidative and anti-apoptotic activity of selegiline, which neuroprotective mechanism may not be related to MAO B inhibition.

Novel MAO B inhibitors as anti-parkinsoian and anti-neurodegenerative drugs

Rasagiline (N-propargyl-1R-aminoindan) is a novel, potent, irreversible MAO B inhibitor designed for use as an anti-perkinsosonian drug. As described above, rasagiline is clinically effective as monotherapy or as an adjunct to L-DOPA for PD (Ives et al., 2004). Youdim et al. (2005) have reported that the neuroprotective activity of rasagiline is associated with the propargylamine moiety, which protects mitochondrial viability and mitochondrial permeability pore by activating Bcl-2 and down-regulating the Bax family of proteins, and that rasagiline processes amyloid precursor protein (APP) into the neuroprotective-neurotrophic soluble APP-alpha by protein kinase C-dependent and mitogen-activated protein kinase-dependent activation of alpha-secretase, and increases expression and proteins of

NGF, GDNF, and BDNF, suggesting its efficacy also in Alzheimer's disease.

Youdim et al. (2004) also reported novel bifunctional drugs targeting MAO inhibition and iron chelation as an approach to neuroprotection in PD and other neurodegenerative diseases. The authors suggest that bi-functional brain penetrable drugs with iron chelating property and MAO inhibitory activity could be the most feasible approach for neuroprotection in neurodegenerative diseases owing to the protection of elevated iron in oxidative stress and also neuroprotective effect by propargylamine moiety.

R-(−)-(Benzofuran-2-yl)-2-propylaminopentane [R-(−)-BPAP], which is a new sensitive enhancer of the impulse propagatin of action potential mediated release of catecholamines and serotonin in the brain (Knoll et al., 1999), was reported to protect apoptosis induced by N-methyl(R)salsolinol, an endogenous DA neurotoxin (Maruyama et al., 2004).

Considering the development of these new neuroprotective drugs, we would be able to expect development of new drugs which are effective against PD, Alzheimer's disease, and various neurodegenerative diseases in preventing or retarding the progress of such diseases.

Neuroprotective effects of MAO B inhibitors and neural growth factors (neurotrophins) and cytokines produced from glial cells in the inflammatory process in Parkinson's disease

Neuroinflammation, especially accompanied by activated microglia in the brain, has been recently noted in PD (for review, see Hirsch et al., 2003; Nagatsu and Sawada, 2005). As the first features of inflammation in PD, McGeer and the collaborators reported an increased number of major histocompatibility complex (MHC) class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive microglial cells in the substantia nigra (McGeer et al., 1988; McGeer and McGeer, 1995). We and other investigators found increased levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha (Mogi et al., 1994a), interleukin (IL)-1beta and IL-6 (Mogi et al., 1994b), and decreased levels of neurotrophins such as BDNF and NGF (Mogi et al., 1999a) in the nigro-striatal region of postmortem brains and/or in the ventricular or lumber cerebrospinal fluid (CSF) from patients with sporadic PD, and in animal models, such as MPTP- and 6-hydroxydopamine-induced PD (for review, see Mogi and Nagatsu, 1999b; Nagatsu et al., 1999, 2000a, b; Nagatsu, 2002a). These changes in cytokine and neurotrophin levels may be initiated by activated microglia, which may then proceed to apoptotic cell death and subsequent phagocytosis of DA neurons.

Cytokines such as IL-6, Il-1beta, or TNF-alpha are pleiotropic factors, and promote signals that either exert neuroprotective effects or neurotoxic effects leading to cell death. Neurotrophins such as BDNF and GDNF are strongly neuroprotective for DA neurons. In order to address the question as to whether microglia activation is neurotoxic or neuroprotective in vivo in PD, we examined activated microglia in the autopsy brain from patients with PD by immunohistochemistry using HLA-DR antibody. We (Imamura et al., 2003) found 2 types of activated microglia, one associated with and one without neuronal degeneration: the former was found in the nigro-striatum; and the latter, in the hippocampus and cerebral cortex. We (Imamura et al., 2005) also observed activated microglia in Lewy body disease (LBD), in which neurodegeneration is observed both in the nigro-striatum and hippocampus (Kosaka, 2002), in the nigro-striatum and hippocampus. In normal controls, neuronal loss and activated microglia were not observed in the hippocampus, and neurons were strongly BDNF-positive. In the hippocampus in PD, BDNF-positive neurons were only slightly decreased. In LBD, the number of activated microglia increased more than those in PD, and all neurons were very weakly stained by anti-BDNF. The results suggest activated microglia in the hippocampus to be probably neuroprotective in PD, but in the nigro-striatum to be neurotoxic. As another evidence supporting this hypothesis, two subsets of microglia were isolated from mouse brain by cell sorting: one subset with high production of ROS and the other with no production of ROS. On the other hand, Sawada with coworkers found that a neuroprotective microglia clone in a co-culture experiment converted to a toxic microglia clone by transduction of the HIV-1 Nef protein with increasing NADPH oxidase activity (Vilhardt et al., 2002). Based on these results, we speculate that activated microglia may change in vivo from neuroprotective to neurotoxic subsets as degeneration of DA neurons in the substantia nigra progresses in PD and that the cytokines from activated microglia in the substantia nigra and putamen may be, at least initially, neuroprotective, but then become neurotoxic during the progress of PD (Sawada et al., 2005).

Another interesting question is the possible interrelationship between familial PD and neuroinflammation. Recent discoveries of the causative genes of familial PD (PARK), starting from discoveries of alpha-synuclein in PARK 1 (Polymeropoulos et al., 1997) and parkin in PARK 2 (Kitada et al., 1998) gave a fresh insight to the molecular mechanism of sporadic PD (for review, see Cookson,

2005). Although the function of alpha-synuclein is not yet clear, alpha-synuclein is a main component of cytoplasmic inclusions called Lewy bodies, which are frequently observed in the residual DA neurons in the substantia nigra in PD. The term Lewy body disease (LBD) is proposed by Kosaka (2002) for neurodegenerative diseases with intracellular Lewy bodies. The parkin gene encodes a ubiquitin ligase E3 (Shimura et al., 2000), and the mutated parkin gene results in a faulty ubiquitin-proteasome system. Since misfolded or unfolded proteins in cells are normally degraded by the ubiquitine-proteasome system, dysfunction of the ubiquitine-proteasome system causes accumulation of misfolded proteins, suggesting that PD as well as other neurodegenerative diseases such as LBD and Alzheimer's disease may also be "protein-misfolding diseases". A puzzling question is that Lewy bodies are not observed in PARK 2. Misfolded substrate proteins of parkin accumulated by loss of function, such as Pael receptor (parkin-associated endothelin receptor-like receptor), which is rich in the nigral region, may accumulate in the endoplasmic reticulum (ER) and cause ER stress (Imai et al., 2001). Although the molecular link is not completely clear, ER stress may cause oxidative stress as observed in idiopathic PD, and may ultimately trigger the cascade of apoptotic cell death. A causal link is speculated between oxidative stress and neuroinflammation in sporadic and familial PD (Hald and Lotharius, 2005).

In another experiment using a primary mesencephalic neuron-glia co-culture system, aggregated alpha-synuclein activated microglia, and microglial activation enhanced DA neurodegeneration induced by aggregated alpha-synuclein depending on phagocytosis of alpha-synuclein and activation of NADPH oxidase with production of ROS (Zhang et al., 2005). NADPH activation in activated microglia agrees with the concept of toxic change of activated microglia proposed by Sawada and coworkers (Vilhard et al., 2002).

In addition to microglia, astrocytes are thought to contribute, although to a lesser extent, to the neurodegenerative process in PD (McNaught and Jenner, 1997). Although astrocytes release neurotrophins or small antioxidants with free radical-scavenging properties (reduced glutathione, ascorbic acid, GDNF, BDNF, NGF, basic fibroblast growth factor (bFGF)), in certain disease conditions they may also produce toxic products such as NO, and pro-inflammatory cytokines (Mena et al., 2002).

Astrocytes contain MAO B (Levitt et al., 1982), but the presence of MAO B in microglia has not been examined yet.

The interrelationship between neuroinflamation and the neuroprotective effects of MAO B inhibitors remains to be further elucidated. However, since selegiline, a MAO B inhibitor, increases the production of neurotrophins like BDNF and NGF probably from glial cells, MAO B inhibitors would be expected to prevent the progress of toxic injury by activated toxic microglia or astrocytes and also the progress of the inflammatory process in PD.

Conclusion

MAO, especially MAO B, may play important roles in the pathogenesis of PD. MAO B inhibitors such as selegiline and rasagiline have been shown to prevent the progress of PD either in combination with L-DOPA or alone (monotherapy). Further study on the mechanism of neuroprotection by MAO B inhibitors would contribute both to elucidation of molecular mechanism of PD and to the development of new neuroprotective drugs against PD which could prevent the onset and progress of PD. Such drug development would also be useful not only against PD but also against Alzheimer's disease and other neurodegenerative diseases.

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Phospholipids modulate superoxide and nitric oxide production by lipopolysaccharide and phorbol 12-myristate-13-acetate-activated microglia

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Abstract

Microglial activation and inflammatory processes have been implicated in the pathogenesis of a number of neurodegenerative disorders. Recently, peroxynitrite ($ONOO^-$), the reaction product of superoxide ($^{\bullet}O_2^-$) and nitric oxide (NO) both of which can be generated by activated microglia, has been demonstrated to act as a major mediator in the neurotoxicity induced by activated microglia. On the other hand, phospholipids such as phosphatidylserine (PS) and phosphatidylcholine (PC) have been reported to modulate the immune function of phagocytes. We therefore evaluated the effects of liposomes which comprise both PS and PC (PS/PC liposomes) or PC only (PC liposomes) regarding the production of both $^{\bullet}O_2^-$ and NO by lipopolysaccharide (LPS)/phorbol 12-myristate-13-acetate (PMA)-activated microglia using electron spin resonance (ESR) spin trap technique with a DEPMPO and Griess reaction, respectively. Pretreatment with PS/PC liposomes or PC liposomes considerably inhibited the signal intensity of $^{\bullet}O_2^-$ adduct associated with LPS/PMA-activated microglia in a dose-dependent manner. In addition, pretreatment with PS/PC liposomes also significantly reduced LPS/PMA-induced microglial NO production. In contrast, pretreatment with PC liposomes had no effect on the NO production. These results indicate that PS/PC liposomes can inhibit the microglial production of both NO and $^{\bullet}O_2^-$, and thus presumably prevent a subsequent formation of $ONOO^-$. Therefore, PS/PC liposomes appear to have both neuroprotective and anti-oxidative properties through the inhibition of microglial activation.

Keywords: Microglia; Superoxide; Nitric oxide; Peroxynitrite; Electron spin resonance; Phosphatidylserine; Phosphatidylcholine

1. Introduction

Increasing evidence shows that microglial activation and inflammatory processes are involved in the pathogenesis of a number of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis and multiple sclerosis (MS) (McGeer et al., 1988; McGeer and McGeer, 2002; Navikas and Link, 1996). In addition, many *in vitro* studies have shown that activated microglia produce an excess of inflammatory and potentially neurotoxic molecules such as pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin 1-β, reactive oxygen species (ROS) and reactive nitrogen species, i.e., superoxide anion ($^{\bullet}O_2^-$) and nitric oxide (NO) and consequently cause neuronal injury or death (Combs et al., 2001; Hashioka et al., 2005; Meda et al., 1995; Qin et al., 2002; Suuronen et al., 2006; Szelenyi et al., 2006). However, the question as to precisely which toxic agent(s) released from

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activated microglia are responsible for neurotoxicity remains unclear.

Recently, peroxynitrite ($ONOO^-$), the reaction product of $^{\bullet}O_2^-$ and NO, has been demonstrated to act as a major mediator in the neurotoxicity induced by amyloid β (Aβ)-activated microglia *in vitro* (Xie et al., 2002). $ONOO^-$ is a highly reactive oxidant capable of inducing injury to a number of cell types (Torreilles et al., 1999). In support of this, $ONOO^-$ generated by lipopolysaccharide (LPS)-activated microglia has been shown to act as a primary cytotoxic factor for oligodendrocytes as well (Li et al., 2005). Therefore, the inhibition of microglial production of both NO and $^{\bullet}O_2^-$ and the subsequent formation of $ONOO^-$ appears to be neuroprotective and a potentially useful treatment for some kinds of neurodegenerative disorder and white matter disorder. $^{\bullet}O_2^-$ is not only a tally of NO for $ONOO^-$ formation but also a limiting factor for $ONOO^-$ formation (Navarro-Antolin et al., 2002; Possel et al., 2002). Furthermore, $^{\bullet}O_2^-$ is a precursor of the other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), and ROS has been reported to mediate pro-inflammatory signaling in activated microglia and thus amplify TNF-α production (Qin et al., 2004). It is, therefore, very important to measure microglial $^{\bullet}O_2^-$ production specifically and directly. Several studies using indirect methods such as cytochrome *c* reduction assay, nitroblue tetrazolium reduction assay and chemiluminescence assay have suggested that activated microglia posses the capacity for $^{\bullet}O_2^-$ production (Colton et al., 1994; Tanaka et al., 1994; Herrera-Molina and von Bernhardi, 2005). So far, however, few studies have directly identified the microglial generation of ROS including $^{\bullet}O_2^-$ using electron spin resonance (ESR) with the spin trap technique (Sankarapandi et al., 1998; Chang et al., 2000). Especially, to our knowledge, there has only been one study, which successfully detected the $^{\bullet}O_2^-$-specific spin adduct formed by activated microglia employing a 5-(diethoxyphosphoryl)-5-methyl-1-pyroline-*N*-oxide (DEPMPO), an apt spin trapping agent for cell-generated $^{\bullet}O_2^-$ (Sankarapandi et al., 1998; Shi et al., 2005).

Phospholipids such as phosphatidylserine (PS) and phosphatidylcholine (PC) have been reported to be able to modulate the immune functions of phagocytes. PC, a major component of the outer leaflet of the plasma membrane, has been demonstrated to reduce the production of ROS and TNF-α in LPS/phorbol 12-myristate-13-acetate (PMA)-activated monocytes (Morris et al., 2000; Tonks et al., 2005). On the other hand, abnormal exposure of PS, which is normally sequestered in the inner leaflet of plasma membrane, in the early phase of apoptosis is an essential determinant for the recognition and ingestion of apoptotic cells by phagocytes (Fadok et al., 1992). After the engulfment of apoptotic cells, macrophages are well known to actively suppress the inflammatory response by releasing anti-inflammatory mediators and thus decreasing the secretion of various pro-inflammatory cytokines (Fadok et al., 1998). Furthermore, PS-containing liposomes have been shown to mimic the effects of apoptotic cells on macrophages (Fadok et al., 2000) and microglia (De Simone et al., 2002; Zhang et al., 2005) through surface molecules that recognize PS.

For the above-mentioned reasons, we evaluated the effects of liposomes comprising both PS and PC (PS/PC liposomes) or PC alone (PC liposomes) on $^{\bullet}O_2^-$ and NO production by LPS/PMA-activated microglia using the ESR spin trap technique with the DEPMPO and Griess reaction, respectively. We herein provide evidence that PS/PC liposomes can inhibit the microglial production of both NO and $^{\bullet}O_2^-$, and thus presumably prevent the subsequent formation of $ONOO^-$.

2. Materials and methods

2.1. Chemicals and reagents

PMA was purchased from Biomol international (Plymouth Meeting, PA, USA). LPS, diethylenetriamine pentaacetic acid (DTPA), and a spintrap DEPMPO were purchased from Sigma Chemicals (St. Louis, MO, USA). Superoxide dismutase (SOD; from bovine erythrocytes, 35,000 U/mg), catalase (from beef liver, 11,500 U/mg), xanthine, and xanthine oxidase were purchased from Wako Pure Chemical Industries (Osaka, Japan). The final concentrations of SOD and catalase correspond to the enzyme activities per volume described in a previous report (Chang et al., 2000). Recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF) was purchased from R&D systems (Minneapolis, MN, USA). Porcine brain derived-L-α-PS, egg derived-L-α-PC, 4-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled PS, and NBD-labeled PC were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Preparation of liposomes

The liposomes were prepared as previously described (Nishikawa et al., 1990). In brief, a mixture of 12 mM PS and 33 mM PC in chloroform was placed in a test tube. The liposomes were composed of either PC only (PC liposomes) or a combination of PS and PC at a molar ratio of 3:7 (PS/PC liposomes). The solvent was removed in a rotary evaporator at 30 °C under reduced pressure and then dried by a desiccator for 1 h. The desiccated lipids were dispersed with a vortex mixer in phosphate buffered saline (PBS) (pH 7.4) to obtain final concentrations of 10 mM total lipids. The lipid suspensions were subsequently sonicated (Tomy UR-20P, Tokyo, Japan) for 10 min on ice. The liposome solutions were centrifuged and then the supernatants were used for the assays. Using either NBD-labeled PS or NBD-labeled PC, NBD-labeled PS/PC liposomes and NBD-labeled PC liposomes were prepared by the same methods as described above.

2.3. Microglial cell cultures

The murine microglial cell line, 6-3, was established from neonatal C57BL/6J(H-2b) mice using a non-enzymatic and non-virus-transformed procedure (Kanzawa et al., 2000). The 6-3 cells closely resemble primary cultured microglia (Kanzawa et al., 2000; Okada et al., 2003). The 6-3 cells were cultured in Eagle's minimal essential medium, 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 μg/ml insulin and 10% fetal calf serum, and maintained at 37 °C with 10% CO_2, 90% air atmosphere. One nanogram per milliliter of mouse recombinant GM-CSF was supplemented in the culture medium to maintain the 6-3 cells because these cells stop to proliferate without it (Kanzawa et al., 2000). The culture media were renewed twice per week.

The primary microglial cells were isolated from mixed cell cultures from the cerebral cortex of 3-day-old Wister rats according to the methods described previously (Hashioka et al., 2005). The medium and culture conditions to maintain the primary microglia were the same as that for 6-3 cells. The culture media were renewed twice per week.

2.4. Fluorescence microscopy

After 3 h treatment of NBD-labeled PS/PC liposomes (100 μM) or NBD-labeled PC liposomes (100 μM), primary cultured rat microglia were mounted

on coverslips at a density of 1.0×10^4 cells/ml and were fixed with 4% paraformaldehyde for 30 min at room temperature. Afterwards, the images were taken at an excitation of 470 nm and an emission of 530 nm by using a fluorescence microscopy (Leica Microsystems DMRB, Wetzlar, Germany).

2.5. ESR spectroscopy

ESR, together with the spin-trapping agent DEPMPO was employed to accurately detect the production of $^{\bullet}O_2^-$ radicals by activated microglia. The 6-3 microglial cells were plated on 12-well tissue culture plates at a density of 1.6×10^6 cells in 400 μl of serum free culture medium per well. The 6-3 cells were incubated 500 ng/ml LPS for 16 h in the presence or absence of pretreatment of PS/PC liposomes or PC liposomes for 1 h at 37 °C. Afterwards, the 6-3 cells were incubated at 37 °C with or without 400 ng/ml PMA for 30 min before beginning the detection of ESR spectra. Cell suspensions (4×10^6 cells/ml) in the culture medium containing 25 mM DEPMPO were transferred to a standard cell capillary, and the ESR measurements were performed at room temperature right after the incubation. The ESR spectra were obtained using a JES-RE1X ESR spectrometer (JEOL, Japan). The setting conditions of the instrument were as follows: magnetic field = 336.7 ± 7.5 mT, modulation amplitude = 2000, modulation width = 0.1 mT, modulation frequency = 100 kHz, time constant = 0.1 s, microwave power = 10 mW, microwave frequency = 9430 MHz and sweep time = 2 min.

2.6. Spin trapping in xanthine/xanthine oxidase sysytem

Xanthine oxidase (0.1 U/ml) was incubated with 0.4 mM xanthine in phosphate–buffer (PB) containing 2 mM DTPA and 20 mM DEPMPO in the presence or absence of 2 mM PS/PC liposomes or PC liposomes. Xanthine oxidase was added last to the mixture to start the reaction. The ESR spectra were recorded at room temperature on a JES-RE1X ESR spectrometer. The setting conditions of the instrument were as follows: magnetic field = 336.7 ± 7.5 mT, modulation amplitude = 500, modulation width = 0.1 mT, modulation frequency = 100 kHz, time constant = 0.03 s, microwave power = 10 mW, microwave frequency = 9430 MHz and sweep time = 1 min.

2.7. NO quantification

The accumulation of NO_2^-, a stable end-product, extensively used as an indicator of NO production by cultured cells, was assayed by the Griess reaction. The 6-3 microglial cells were plated on 24-well tissue culture plates at 9×10^5 per 200 μl per well and incubated in the presence or absence of pretreatment of PS/PC liposomes or PC liposomes for 1 h at 37 °C. Afterwards, the cells were incubated in the presence or absence of 500 ng/ml LPS and 400 ng/ml PMA at 37 °C. After 48 h, the cell-free supernatants were mixed with equal amounts of Griess reagent (Griess Reagent Kit; Dojindo, Kumamoto, Japan). Samples were incubated at room temperature for 15 min and subsequently absorbance was read at 540 nm using a plate reader (Multiskan MS; Labsystems, UK).

2.8. Statistics

The values were expressed as the means ± S.E.M. and analyzed by a one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. The significance was established at a level of $p < 0.05$.

3. Results

3.1. Confirmation of microglial phagocytosis of PS/PC liposomes

First, in order to confirm that the PS/PC liposomes are certainly engulfed by microglia, we treated primary cultured rat microglia with NBD-labeled PS/PC liposomes or NBD-labeled PC liposomes. After 3 h of treatment, microglia were fixed with 4% PFA and examined by fluorescence microscopy. The florescent images were merged with the corresponding phase contrast images. As shown in Fig. 1A, it was observed that well-defined microglial cytoplasm was filled with florescently labeled PS/PC liposomes (green). In contrast, few PC liposomes labeled with the florescence were observed in microglial cytoplasm (Fig. 1B). These findings indicate that PS/PC liposomes, but not PC liposomes, were phagocytosed by microglia.

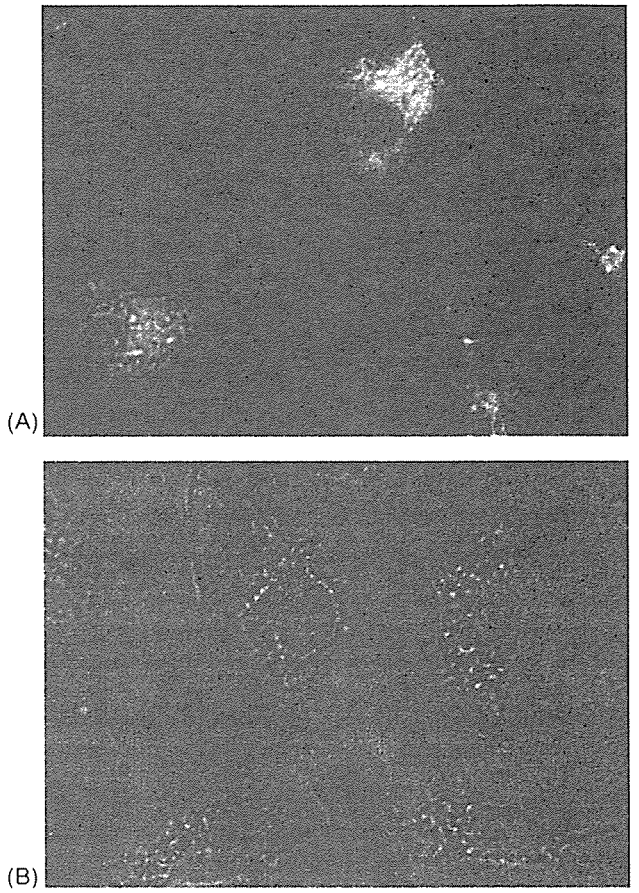

Fig. 1. Microglial phagocytosis of PS/PC liposomes. A typical fluorescence microphotograph showing (A) phagocytosis of NBD-labeled PS/PC liposomes (green) by primary cultured rat microglia and (B) little phagocytosis of NBD-labeled PC liposomes by primary cultured rat microglia.

3.2. Effect of the liposomes on the $^{\bullet}O_2^-$ production by LPS/PMA-activated microglia

We subsequently measured the generation of $^{\bullet}O_2^-$ associated with activated microglia by ESR monitoring with a spin trap DEPMPO. In the preparations of non-stimulated microglia (Fig. 2A) and 500 ng/ml LPS alone-stimulated microglia (Fig. 2B), no signals were obtained. Microglial cells stimulated by 500 ng/ml LPS combined with 400 ng/ml PMA in the presence of 25 mM DEPMPO showed the prominent signals whose spectrum consisting of a linear combination of a characteristic 12-line spectrum corresponding to $^{\bullet}O_2^-$ spin

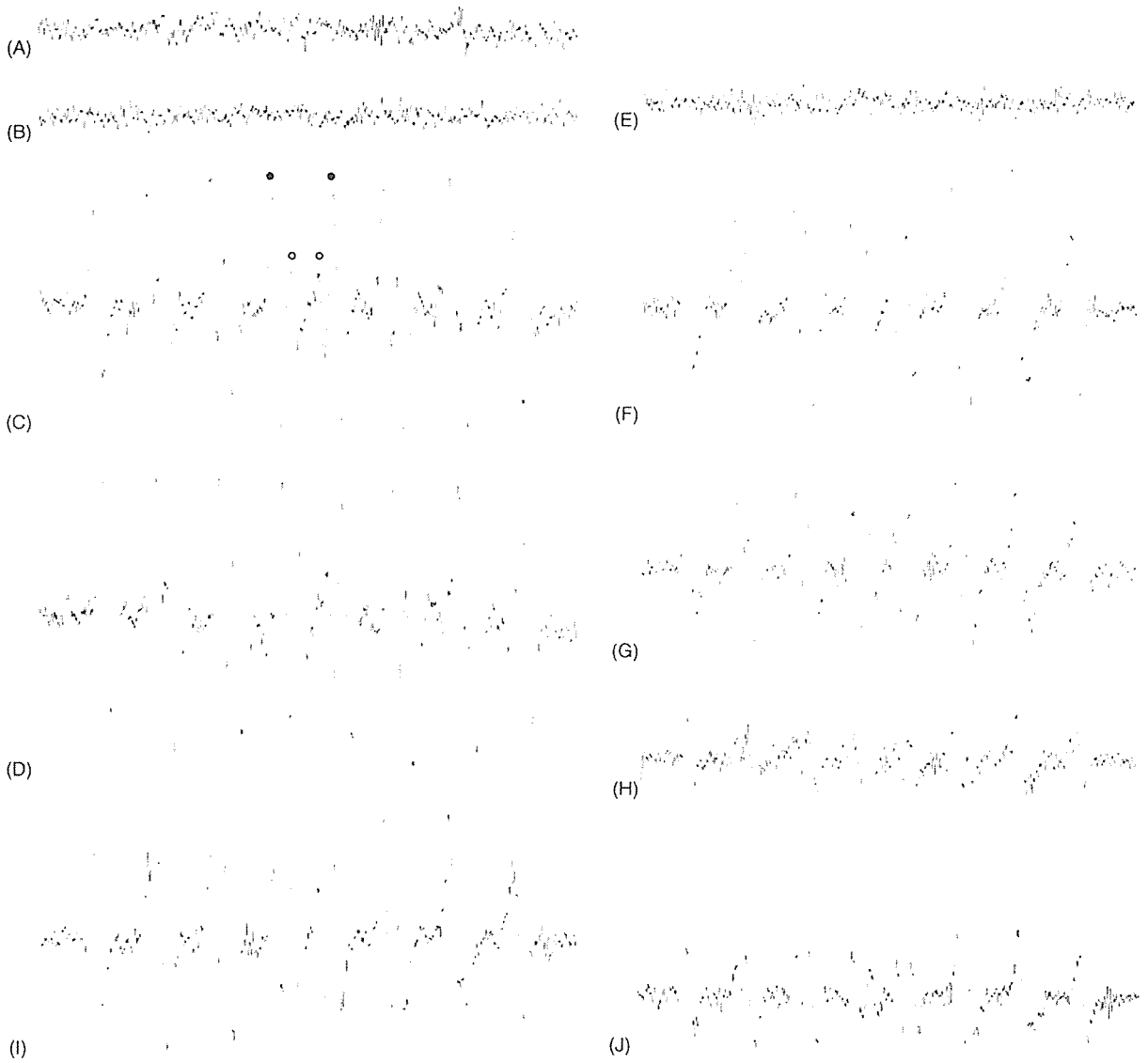

Fig. 2. Detection of $^{\bullet}O_2^-$ generation by LPS/PMA-activated microglia using ESR spin trap technique with DEPMPO. 6-3 microglial cells (4×10^6/ml) were incubated with LPS (500 ng/ml) for 16 h and PMA (400ng/ml) for 30 min at 37 °C with or without pretreatment of PS/PC liposomes for 1 h. The ESR spectra were then recorded in the presence of 25 mM DEPMPO at room temperature. (A) ESR spectra of DEPMPO adducts obtained from non-stimulated microglia. (B) ESR spectra of DEPMPO adducts obtained from microglia stimulated by LPS (500 ng/ml) alone. (C) ESR spectra of DEPMPO adducts obtained from LPS/PMA-activated microglia. Open and closed circles represent measured signal peaks of DEPMPO–OH and DEPMPO–OOH adducts, respectively. (D) ESR spectra of DEPMPO adducts obtained from microglia stimulated by PMA (400 ng/ml) alone. (E) the same as (C), but with the addition of SOD (160 μg/ml). (F) the same as (C), but with the addition of catalase (280 μg/ml). (G) the same as (C), but after pretreatment with PS/PC liposomes (0.1 mM) for 1 h. (H) the same as (C), but after pretreatment with PS/PC liposomes (1 mM) for 1 h. (I) the same as (C), but after pretreatment with PC liposomes (0.1 mM) for 1 h. (J) the same as (C), but after pretreatment with PC liposomes (1 mM) for 1 h.

adduct DEPMPO–OOH and an eight-line spectrum corresponding to $^{\bullet}OH$ spin adduct DEPMPO–OH (Fig. 2C). Computer simulation confirmed DEPMPO–OOH with hyperfine splittings $a_N = 13.15$ G, $a_H^{\beta} = 10.59$ G, $a_p = 49.73$ G, $a_H^{\gamma} = 0.72$ G and DEPMPO–OH with hyperfine splittings $a_N = 12.43$ G, $a_H = 13.49$ G, $a_p = 50.39$ G. These values are consistent with those described in a previous report (Sankarapandi et al., 1998). In addition, microglial cells stimulated by 400 ng/ml PMA alone showed essentially the same ESR spectra as those of LPS/PMA-activated microglia (Fig. 2D). To further confirm the original species of the spin adduct generated by LPS/PMA-activated microglia, SOD (160 μg/ml) or catalase (280 μg/ml) were also treated. The ESR signal intensity was substantially decreased by SOD (Fig. 2E), not by catalase (Fig. 2F). These results indicate that the spin adducts originated from $^{\bullet}O_2^-$ radical but not $^{\bullet}OH$ radical, which is derived from H_2O_2.

We next evaluated the effect of the liposomes on the generation of $^{\bullet}O_2^-$ associated with LPS/PMA-activated microglia. Pretreatment with PS/PC liposomes for 1 h considerably

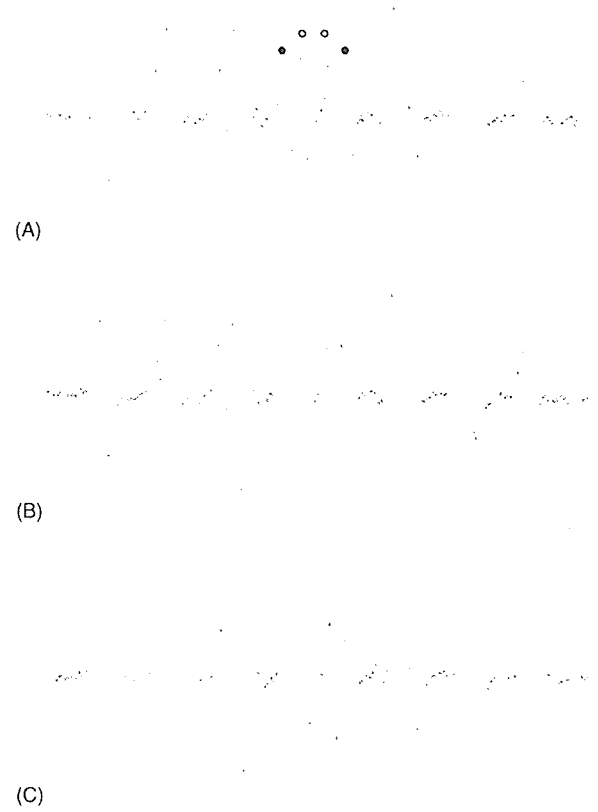

Fig. 3. Detection of $^{\bullet}O_2^-$ generation in xanthine/xanthine oxidase system using ESR spin trap technique with DEPMPO. The system contained 0.4 mM xanthine, 2 mM DTPA, and 20 mM DEPMPO in PB in the presence or absence of 2 mM PS/PC liposomes or PC liposomes. Xanthine oxidase (0.1 U/ml) was added last to the mixture to start the reaction. (A) ESR spectra of DEPMPO adducts obtained in the xanthine/xanthine oxidase system. Open and closed circles represent measured signal peaks of DEPMPO–OH and DEPMPO–OOH adducts, respectively. (B) the same as (A), but in the presence of 2 mM PS/PC liposomes. (C) the same as (A), but in the presence of 2 mM PC liposomes.

inhibited the signal intensity of the $^{\bullet}O_2^-$ adduct in a dose-dependent manner (Fig. 2G, H). In spite of few PC liposomes were phagocytosed by microglia, pretreatment with PC liposomes for 1 h also inhibited the signal intensity of the $^{\bullet}O_2^-$ adduct in a dose-dependent manner (Fig. 2I, J).

3.3. Effect of the liposomes on the $^{\bullet}O_2^-$ generation in xanthine/xanthine oxidase system

To confirm whether or not the liposomes *per se* scavenge $^{\bullet}O_2^-$, we measured the $^{\bullet}O_2^-$ production in xanthine/xanthine oxidase system in the presence or absence of the liposomes by ESR monitoring with a spin trap DEPMPO. Fig. 3A shows typical ESR spectra consisting of DEPMPO–OOH and DEPMPO–OH in xanthine/xanthine oxidase system. The formation of these spin adducts via trapping $^{\bullet}O_2^-$ was confirmed by experiments in which SOD (160 μg/ml) was added before xanthine oxidase and ESR signals were completely quenched (data not shown), while catalase

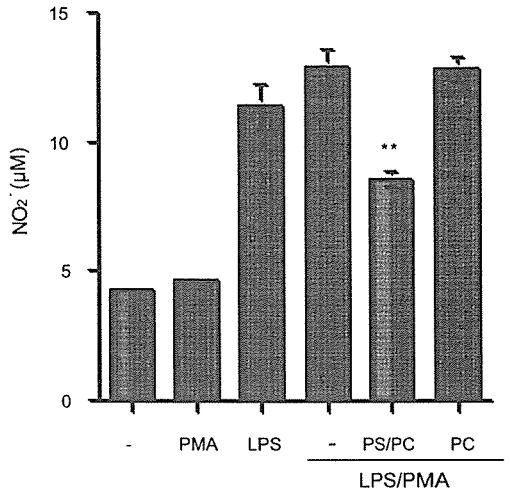

Fig. 4. Effect of the liposomes on the NO production by LPS/PMA-activated microglia. 6-3 microglial cells were incubated with LPS (500 ng/ml) and PMA (400 ng/ml) at 37 °C with or without pretreatment with 1 mM PS/PC liposomes or PC liposomes for 1 h. After 48 h, collected media were assayed for NO accumulation using the Griess reaction. $^{**}p < 0.01$, compared with LPS/PMA. The data are the mean values ± S.E.M. ($n = 3$).

(280 μg/ml) was added, in which ESR signals were not quenched at all (data not shown). The ESR spectra in the presence of either 2 mM PS/PC liposomes or PC liposomes showed essentially the same as those shown in Fig. 3A, thus indicating that neither PS/PC liposomes nor PC liposmes have scavenging effect on $^{\bullet}O_2^-$, but have the inhibitory effect on the $^{\bullet}O_2^-$-generating system in microglia (Fig. 3B, C).

3.4. Effect of the liposomes on the NO production by LPS/PMA-activated microglia

Using a Griess reaction assay, we investigated the effect of the liposomes on the microglial production of NO, a tally of $^{\bullet}O_2^-$ for forming $ONOO^-$. The incubation of microglial cells with 500 ng/ml LPS combined with 400 ng/ml PMA for 48 h resulted in a significant elevation in the accumulation of nitrite (Fig. 4). Five hundred nanograms per milliliters LPS alone, also, showed nearly the same amount of microglial production of NO, whereas, 400 ng/ml PMA alone could not induce a significant increase of the NO production. As expected, the LPS/PMA-induced microglial NO production significantly decreased after pretreatment with 1 mM PS/PC liposomes for 1 h (Fig. 4). In contrast, pretreatment with 1 mM PC liposomes for 1 h did not affect the microglial NO production at all (Fig. 4).

4. Discussion

In the present study, PS/PC liposomes considerably inhibited both $^{\bullet}O_2^-$ and NO production in LPS/PMA-activated microglia and thus presumably prevent the subsequent formation of $ONOO^-$, a powerful oxidant capable of inducing strong toxicity. $ONOO^-$ is formed from non-enzymatic reaction between $^{\bullet}O_2^-$ and NO at the near diffusion-limited

rate whose constant is three times faster than rate at which superoxide dismutase scavenges $^{\bullet}O_2^-$ (Beckman, 1994; Torreilles et al., 1999). $ONOO^-$, therefore, can be generated in several regions *in vivo* where $^{\bullet}O_2^-$ and NO are produced simultaneously, as it is presumed to occur in central nervous system. Indeed, the levels of nitrotyrosine, which is a product of the reaction of $ONOO^-$ with tyrosine residues and considered as a permanent footprint of $ONOO^-$, have been reported to increase in AD, PD and MS brains (Torreilles et al., 1999; Liu et al., 2001). Accordingly, the $ONOO^-$ forming blockers including PS/PC liposomes seem to be neuroprotective and a potentially useful treatment for these neurodegenerative diseases.

Due to the pivotal role of $^{\bullet}O_2^-$ in microglia-mediated neuroinflammation and oxidative stress, we employed ESR with the spin trap technique using DEPMPO to directly measure microglial $^{\bullet}O_2^-$ generation. The DEPMPO is an appropriate spin-trapping agent for cell-generated-$^{\bullet}O_2^-$ detection because of its stability and capability of differentiating between $^{\bullet}O_2^-$ and $^{\bullet}OH$ (Shi et al., 2005; Mojovic et al., 2005). LPS/PMA-activated microglia gave rise to ESR spectra consisting of a linear combination of $^{\bullet}O_2^-$ spin-adduct DEPMPO–OOH and spin-adduct DEPMPO–OH. Radical generation was totally quenched by SOD but not by catalase, thus indicating that H_2O_2, which is reduced to $^{\bullet}OH$ by Fenton reaction in the presence of Fe^{2+} or Zn^{2+}, was not a significant reactant in the formation of the observed radial signals. Moreover, the DEPMPO–OH appears to be generated by a spontaneous reduction of DEPMPO–OOH, not from H_2O_2-derived $^{\bullet}OH$ (Mojovic et al., 2005).

Colton et al. (1994) have reported that $^{\bullet}O_2^-$ and NO are apparently not produced by the same activating agent in rat primary cultured microglia. LPS is one of the most common stimulators used to activate microglia (O'Shea et al., 2006; Suuronen et al., 2006) both *in vivo* and *in vitro* models of neuroinflammation-mediated neurodegeneration and is known to activate protein–tyrosine kinases, mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), which have been implicated in the release of NO and various pro-inflammatory cytokines (Rivest, 2003; Suuronen et al., 2006; Szelenyi et al., 2006). On the other hand, PMA assembles NADPH oxidase via activation of protein kinase C (PKC) and thus is commonly used to induce abrupt and large amounts of microglial $^{\bullet}O_2^-$ production called as respiratory burst. Although several studies have demonstrated that microglia activated by LPS can form $^{\bullet}O_2^-$ (Qin et al., 2004, 2005), our study demonstrated that LPS alone did not affect the microglial $^{\bullet}O_2^-$ production but it did induce NO production. In contrast, PMA alone induced microglial $^{\bullet}O_2^-$ generation without affecting the NO production. In addition, according to our findings, and consistent with the study by Colton et al., it is suggested that $^{\bullet}O_2^-$ and NO production are differentially regulated in cultured murine microglial cells. Namely, LPS appears to be an inflammogen that provokes microglial production of NO rather than $^{\bullet}O_2^-$. We cannot, however, eliminate the possibility that LPS stimulation mediates the pathway(s) associated with $^{\bullet}O_2^-$ generation in microglia, because of differences on $^{\bullet}O_2^-$ detection sensitivity between the ESR assay and the other indirect methods.

We demonstrated that PS/PC liposomes considerably inhibited both the $^{\bullet}O_2^-$ and NO production in LPS/PMA-activated microglia, even though both of them appear to be regulated by distinct signal pathways. Furthermore, we also demonstrated that PS/PC liposomes did not scavenge $^{\bullet}O_2^-$, but instead act on the $^{\bullet}O_2^-$-generating system in microglia. The exact mechanism of PS/PC liposomes to suppress inflammatory activation of microglial has not yet been elucidated. Concerning $^{\bullet}O_2^-$ production, not only PS/PC liposomes but also PC liposomes inhibited the LPS/PMA-induced microglial production. Dipalmitoyl PC (DPPC), a kind of PC and the major component of pulmonary surfactant, has been shown to reduce monocyte respiratory burst via the downregulation of PKC associated with plasma membrane by the presumed mechanism that DPPC induces membrane perturbation (Tonks et al., 2005). In addition to that, according to our finding that few PC liposomes were phagocytosed by the micrglia, PC liposomes seem to act as membrane perturbers, thus reducing the LPS/PMA-induced and PKC-mediated $^{\bullet}O_2^-$ production. In contrast, PS has been reported to be engulfed by phagocytes through PS-recognizing receptor such as PS receptor (Fadok et al., 2000; De Simone et al., 2002) and scavenger receptor class B type 1 (Zhang et al., 2005). Indeed, it is confirmed that PS/PC liposomes could be phagocytosed by microglia in our study. However, we cannot rule out the possibility that PS/PC liposomes also induced the membrane perturbation under our experimental conditions because PS/PC liposomes contain 70 molar% PC. On the other hand, Ajmone-Cat et al. (2003) demonstrated that PS/PC-containing liposomes inhibited the phosphorylation of p38 MAPK and delayed that of cAMP responding element-binding protein in LPS-activated microglia. Because phosphorylation of p38 MAPK has been shown to mediate the signal pathway reacting for inflammatory stimulants and result in gene induction of NO synthase in microglia (Koistinaho and Koistinaho, 2002), the PS/PC liposomes-induced inhibition of p38 MAPK phosphorylation in activated microglia appears to suppress, at least partially, NO generation. In contrast, our finding that PC liposomes had no effect on LPS/PMA-induced NO production indicates that the presumed membrane perturbation induced by PC liposomes is not involved in NO-generating pathway(s) in the LPS/PMA-activated microglia.

Several neuroprotective compounds such as isoproterenol, dexamethasone, nicergoline, and naloxone have been shown to suppress microglial activation and thus decrease the ROS generation (Colton and Chernyshev, 1996; Yoshida et al., 2001; Qin et al., 2005). In comparison to the effective concentrations of these compounds (e.g. isoproterenol and nicergoline act at micromolar, naloxone acts even at femtomolar), the total lipids concentration such as millimolar of PS/PC liposomes used in this study seems to certainly be high. Borisenko et al. (2003), however, have suggested that phagocytes have a sensitivity threshold for PS externalized on the target cell surface, which thus provides for the reliable recognition and distinction between normal cells with low amounts of externalized PS and