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-perkinsososonian 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.

Aknowledgements

We dedicate this paper to Prof. Dr. Moussa Youdim in celebrating his great contribution to basic research on MAO and PD and to development of MAO B inhibitors as new drugs, which are expected to have neuroprotective efficacy to PD, Alzheimer's disease, and other neurodegenerative diseases.

We are grateful to our collaborators in the study of cytokines and microglia in PD: Drs. M. Mogi (Aichi Gakuin University), K. Imamura (Okazaki City Hospital), Y. Hashizume (Aichi Medical University), and Peter Riederer (Wuerzburg University, Wuerzburg), and their collaborators.

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Neurochemistry International 50 (2007) 499–506

NEUROCHEMISTRY International

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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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Received 7 April 2006; received in revised form 12 October 2006; accepted 19 October 2006
Available online 28 November 2006

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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0197-0186/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2006.10.006

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.

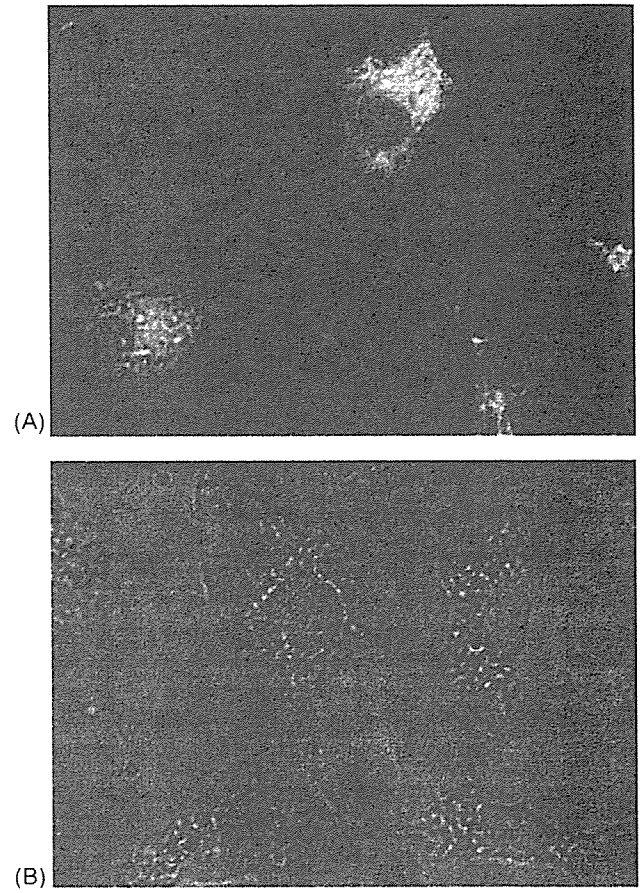

Fig. 1. Microglial phagocytosis of PS/PC liposomes. A typical fluorescence microphotograph showing (A) phagocytosis of NBD-labeled PS/PC liposomes (green) by primary cultured rat microglia and (B) little phagocytosis of NBD-labeled PC liposomes by primary cultured rat microglia.

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

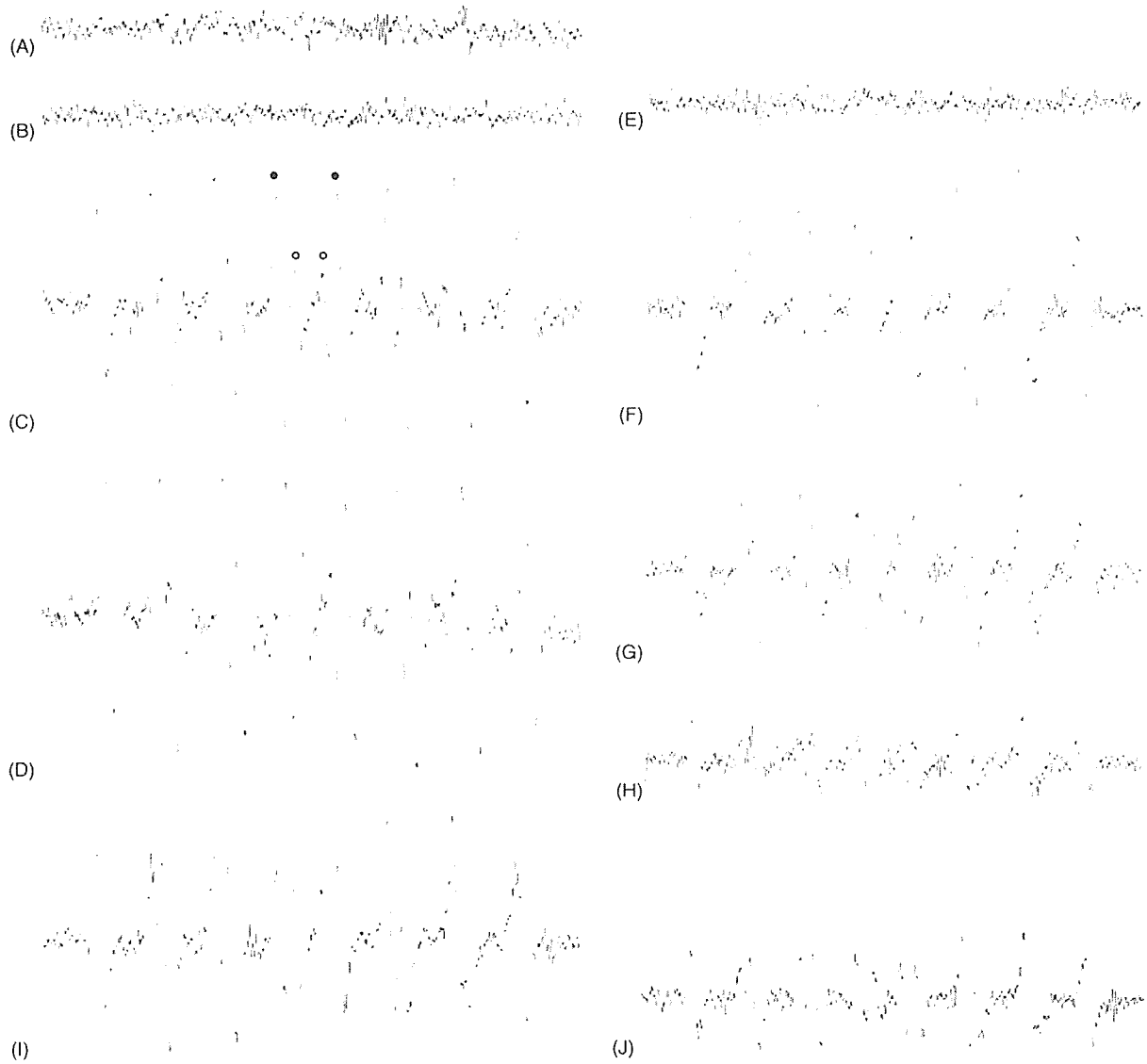

Fig. 2. Detection of $^{\bullet}O_2^-$ generation by LPS/PMA-activated microglia using ESR spin trap technique with DEPMPO. 6-3 microglial cells (4×10^6/ml) were incubated with LPS (500 ng/ml) for 16 h and PMA (400ng/ml) for 30 min at 37 °C with or without pretreatment of PS/PC liposomes for 1 h. The 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

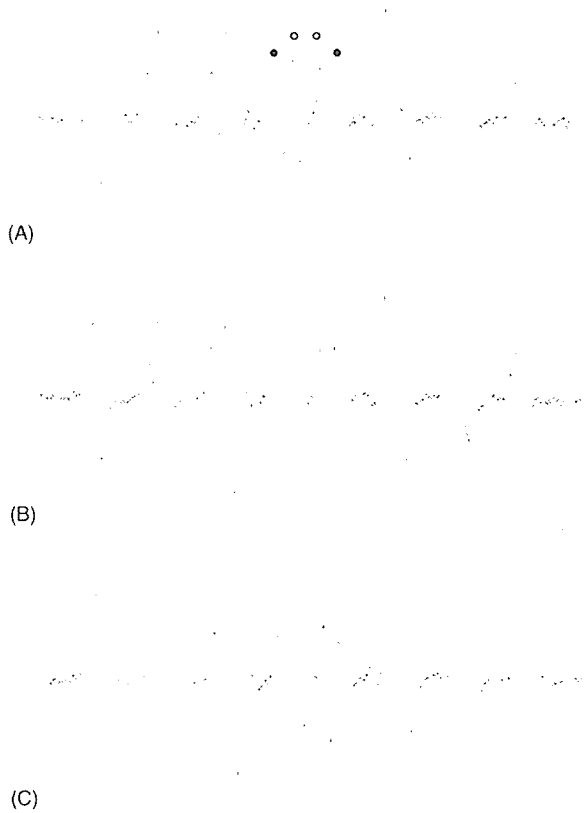

Fig. 3. Detection of $^{\bullet}O_2^-$ generation in xanthine/xanthine oxidase system using ESR spin trap technique with DEPMPO. The system contained 0.4 mM xanthine, 2 mM DTPA, and 20 mM DEPMPO in PB in the presence or absence of 2 mM PS/PC liposomes or PC liposomes. Xanthine oxidase (0.1 U/ml) was 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

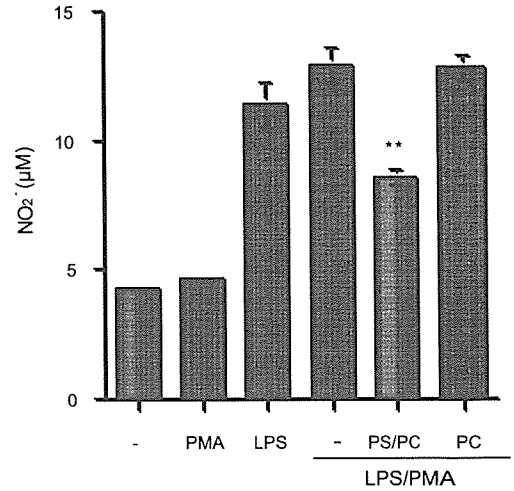

Fig. 4. Effect of the liposomes on the NO production by LPS/PMA-activated microglia. 6-3 microglial cells were incubated with LPS (500 ng/ml) and PMA (400 ng/ml) at 37 °C with or without pretreatment with 1 mM PS/PC liposomes or PC liposomes for 1 h. After 48 h, collected media were assayed for NO accumulation 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

apoptotic cells with remarkably elevated PS levels. They estimated that, using the liposomes containing PS and PC (1:1), the absolute amounts of PS required for phagocytosis by 5×10^4 macrophages (the threshold of macrophage sensitivity) was 7 pmol. This value of the PS amount for 10^6 macrophages (i.e. 140 pmol) approximates to the normalized value of the PS amount for 10^6 microglial cells, which was found to be considerably effective in our study (i.e. 75 pmol). Taken together, these findings suggest that microglia also require the relatively high phospholipids concentration to recognize PS/PC liposomes as apoptotic cells. Accordingly, new techniques to ameliorate the stability of PS/PC liposomes and thus reduce the effective phospholipids concentration are called for based on the findings of *in vivo* studies.

Acknowledgements

This study was supported by a grant from Inogashira Hospital (SH). We thank Prof. Yukihiro Shoyama and Dr. Satoshi Morimoto, Department of Plant Resources Regulation, Graduate School of Pharmaceutical Sciences, Kyushu University, for technical advice about preparing liposomes.

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Original Contribution

Phosphatidylserine and phosphatidylcholine-containing liposomes inhibit amyloid β and interferon-γ-induced microglial activation

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Received 21 September 2006; revised 16 November 2006; accepted 5 December 2006
Available online 9 December 2006

Abstract

There is increasing evidence that microglial activation is one of the major pathogenic factors for Alzheimer's disease (AD) and the inhibition of the inflammatory activation of the microglia thus appears to be neuroprotective and a potentially useful treatment for AD. Phospholipids such as phosphatidylserine (PS) and phosphatidylcholine (PC) have been reported to modulate the immune function of phagocytes. In addition, PS has been reported to be a nootropics that can be used as nonprescription memory or cognitive enhancers. We therefore evaluated the effects of liposomes, which comprise both PS and PC (PS/PC liposomes), on the microglial production of tumor necrosis factor-α (TNF-α), nitric oxide (NO), and superoxide ($^{\bullet}O_2^-$) induced by amyloid β (Aβ) and interferon-γ (IFN-γ). Pretreatment of microglia with PS/PC liposomes considerably inhibited the TNF-α, NO and $^{\bullet}O_2$ production induced by Aβ/IFN-γ. These results suggest that PS/PC liposomes have both neuroprotective and antioxidative properties through the inhibition of microglial activation, thus supporting the nootropic and antidementia effect of PS.

Keywords: Alzheimer's disease; Microglia; Phosphatidylserine; Phosphatidylcholine; Nitric oxide; Superoxide; Peroxynitrite; Electron spin resonance

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease known to cause progressive memory loss and confusion. Recently, much attention has been paid to microglial activation as a major pathogenic factor for AD. Senile plaque is the site of inflammatory processes, as evidenced by the presence of degenerating neurons and numerous reactive microglia and astrocytes associated with such plaques [1]. Microglial activation has been reported to be a relatively early pathogenetic event which precedes the process of neuropil destruction in AD patients [2]. According to in vitro studies, the microglia activated by amyloid β peptides (Aβ) have been well reported to damage or kill neurons by the excessive release of inflammatory and potentially neurotoxic molecules such as proinflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), intrleukin-1β], reactive oxygen species (ROS), and reactive nitrogen species

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyroline-*N*-oxide; ESR, electron spin resonance; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NADPH oxidase, nicotinamide adenine diphosphate reduced form oxidase; NO, nitric oxide; PS, phosphatidylserine; PC, phosphatidylcholine; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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doi:10.1016/j.freeradbiomed.2006.12.003

[i.e., superoxide radical ($^{\bullet}O_2^-$) and nitric oxide (NO), respectively] [3–6]. Peroxynitrite ($ONOO^-$), the reaction product of $^{\bullet}O_2^-$ and NO, is a highly reactive oxidant and has recently been demonstrated to act as a major mediator in the neurotoxicity induced by Aβ-activated microglia in vitro [7]. 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 brains [8]. Taken together, the inhibition of Aβ-induced microglial production of inflammatory cytokines including TNF-α and free radicals such as NO, $^{\bullet}O_2^-$, and subsequent formation of $ONOO^-$ appears to be neuroprotective and a potentially useful treatment for AD.

Phospholipids such as phosphatidylserine (PS) and phosphatidylcholine (PC) have been shown to modulate the immune functions of phagocytes. PC, a major component of the outer leaflet of plasma membrane, has been demonstrated to reduce the production of ROS in lipopolysaccharide (LPS)/phorbol 12-myristate-13-acetate (PMA)-activated monocytes [9]. 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 [10]. After the engulfment of apoptotic cells, macrophages are well known to actively suppress the inflammatory response through the release of anti-inflammatory mediators, thereby decreasing the secretion of various proinflammatory cytokines [11]. Furthermore, PS-containing liposomes have been shown to mimic the effects of apoptotic cells on macrophages [12] and on microglia [13,14] through surface molecules that recognize PS. Intriguingly, PS has also been reported to be one of the nootropics that can be used as nonprescription memory or cognitive enhancers [15]. According to in vivo studies, PS treatment has been demonstrated to ameliorate the impaired functions of learning and memory on a variety of tasks in aged rats [16] and PS-containing liposomes have been shown to protect LPS-induced impairment of long-term potentiation (LTP) in adult rats [17]. In human clinical trials, some studies have shown that the oral administration of bovine brain cortex-derived PS improves the behavior and cognitive performances of patients with senile dementia [18,19].

For the above-noted reasons, we evaluated the effects of liposomes comprising PS and PC on the Aβ and interferon-γ (IFN-γ)-induced microglial production of proinflammatory molecules such as TNF-α, NO, and $^{\bullet}O_2^-$. Especially, $^{\bullet}O_2^-$ is a key molecule in the oxidative stress involved in the pathogenesis of AD because $^{\bullet}O_2^-$ is not only a tally of NO for $ONOO^-$ formation but also a limiting factor for $ONOO^-$ formation [20,21]. Furthermore, $^{\bullet}O_2^-$ is a precursor of other types of ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), and ROS has also been reported to mediate proinflammatory signaling in activated microglia, thereby amplifying the TNF-α production [22]. We, therefore, measured the microglial $^{\bullet}O_2^-$ production specifically and directly using electron spin resonance (ESR) with the spin-trap technique. To our knowledge, this is the first report to directly trap $^{\bullet}O_2^-$ associated with Aβ-stimulated microglia.

Materials and methods

Aβ peptides

The Aβ25–35 peptides used in this study were purchased from AnaSpec (San Jose, CA). Purity was certified by high-performance liquid chromatography–mass spectrometry for each of the peptide. The peptides were resuspended in sterile double-deionized water, aliquoted at 5 mg/ml, and kept at −20°C. We preliminarily confirmed that neither PS/PC liposomes (data not shown) nor IFN-γ [4] affected the amyloid fibril structure of the Aβ25–35 peptides using a thioflavine-T fluorometric assay.

Chemicals and reagents

A spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyroline-*N*-oxide (DEPMPO), LPS, and diethylenetriamine pentaacetic acid (DTPA) were purchased from Sigma Chemicals (St. Louis, MO). Superoxide dismutase (SOD; from bovine erythrocytes, 3500 U/mg), catalase (from beef liver, 11500 U/mg), xanthine, and xanthine oxidase were purchased from Wako (Osaka, Japan). The final concentrations of SOD and catalase correspond to the enzyme activities per volume as described in a previous report [23]. Recombinant IFN-γ was purchased from Genzyme (Cambridge, MA). Recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF) was purchased from R&D systems (Minneapolis, MN). 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).

Preparation of liposomes

The liposomes were prepared as previously described [24]. 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 a combination of PS and PC at a molar ratio of 3:7 (PS:PC) (PS/PC liposomes) or PC only (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 a final concentration 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.

Microglial cell cultures

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 [4,25]. The cerebral cortex was minced and treated with papain (90 U/ml) and DNase (2000 U/ml) at 37°C for 15 min. The mechanically

dissociated cells were gently passed through plastic tips and seeded into plastic flasks at a density of 10^7/300 cm^2 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. The subsequent medium replacement was renewed twice per week. After 10–14 days of culture, floating cells and weakly attached cells on the primary cultured cell layer were isolated by gently shaking the flask for 10 min. The resulting cell suspension was transferred to a petri dish (Falcon 1001, Lincoln Park, NJ) and then allowed to adhere at 37°C. The unattached cells were removed after 25 min, and microglial cells were isolated as strongly adhering cells. About 90% of these attached cells were positive for OX42 (Serotec Ltd., Bichester, UK), a marker for macrophage/microglal cell types.

The murine microglial cell line, 6-3, was established from neonatal C57BL/6J(H-2b) mice using a nonenzymatic and non-virus-transformed procedure [26]. The 6-3 cells closely resemble primary cultured microglia [26,27]. 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 then maintained at 37°C with 10% CO_2, 90% air atmosphere. The amount of 1 ng/ml mouse recombinant GM-CSF was supplemented in the culture medium to maintain the 6-3 cells because these cells stopped proliferating without it [26]. The culture media were renewed twice per week.

Fluorescence microscopy

After the 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. Afterward, the images were taken at an excitation of 470 nm and an emission of 530 nm using fluorescence microscopy (Leica Microsystems DMRB, Wetzlar, Germany).

TNF-α quantification

Primary cultured rat microglial cells were plated on 96-well tissue culture plates at 6×10^4 per 150 μl per well and then were incubated in the presence or absence of 50 μM Aβ25–35 peptides and/or 100 U/ml IFN-γ at 37°C. After 24 h, the collected media were assayed for TNF-α accumulation. TNF-α released into the culture medium was measured using a rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA) based on the quantitative "sandwich" enzyme immunosorbent technique. The assay was carried out according to the manufacturer's protocol. The sensitivity of this assay was 4 pg/ml.

RNA isolation and semiquantitative RT-PCR

Primary cultured rat microglial cells were plated on 60-mm dishes at a density of $1.0–1.5 \times 10^6$ cells per dish and then incubated with stimuli for 4 h. Cellular mRNA from microglia was extracted and purified by a QuickPrep micro mRNA purification kit (Amersham, UK) according to the manufacturer's protocol. The mRNA was subsequently reverse-transcribed to single-stranded complimentary DNA (cDNA) by SuperScrip II (Invitrogen, Carlsbad, CA) with the gene-specific primers described below. Aliquots of the cDNAs were then used in separate PCR amplifications using *Taq* polymerase (Invitrogen). To determine the optimal conditions which allowed the signal to be in the liner portion of the amplification curve, experiments were performed under conditions in which the cycle number and template concentrations were altered. A negative control lacking the template RNA or reverse transcriptase was included in each experiment. The cDNA products of the reverse transcription reaction were denatured at 94°C for 2 min and then PCR amplifications were carried out as follows: for TNF-α (primers, sense 5′-CCC AGA CCC TCA CAC TCA GAT-3′; antisense 5′-TTG TCC CTT GAA GAG AAC CTG-3′), 35 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s; for GAPDH (primers, sense 5′-ACC ACA GTC CAT GCC ATC AC-3′; antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′) as an internal standard, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. PCR products were separated electrophoretically on 2% agarose gel and stained with 0.1 μg/ml ethidium bromide, and then were subsequently visualized under UV illumination. The results of a scanning densitometric analysis (NIH Image 1.62 and Adobe Photoshop 7.0) are expressed as the relative ratio of TNF-α/GAPDH.

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. Primary cultured rat microglial cells were plated on 96-well tissue culture plates at 6×10^4 per 150 μl per well and incubated in the presence or absence of 50 μM Aβ25–35 peptides and 100 U/ml IFN-γ at 37 °C. After 72 h, the cell-free supernatants were mixed with equal amounts of Griess reagent (Dojindo, Kumamoto, Japan). Samples were incubated at room temperature for 15 min and subsequently absorbance was read at 540 nm using a plate reader (Model 550; Bio-Rad, Richmond, CA).

Western blotting for the detection of inducible NO synthase (iNOS)

6-3 microglial cells were plated on 6-well tissue culture plates at a density of $1.0–1.5 \times 10^6$ cells per well and then incubated with stimuli for 12 h. Afterward, cells were washed with PBS (pH 7.4) and lysed with sodium dodecyl sulfate (SDS)-containing sample buffer. Proteins were separated in a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The membrane was incubated with 5% nonfat dry milk to block nonspecific binding. Subsequently, the membrane was incubated with iNOS antibodies (1:2000, Upstate, Lake Placid, NY) and β-actin antibodies (1:1000, Abcam, Cambridge, MA). The expression of iNOS was detected using an enhanced chemiluminescence

system (Amersham). The band intensity was quantified with a densitometric scanner (LAS 1000, Fujifilm, Tokyo, Japan).

ESR spectroscopy

ESR, together with the spin-trapping agent DEPMPO was employed to accurately detect the production of $^{\bullet}O_2^-$ by activated microglia. The 6-3 microglial cells were cultured on 12-well tissue culture plates at a density of 2×10^6 cells in 400 μl of culture medium per well. The 6-3 cells were primed by 100 U/ml IFN-γ for 16 h in the presence or absence of the pretreatment with PS/PC or PC liposomes for 1 h at 37°C. Afterward, the 6-3 cells were incubated at 37°C with or without 50 μM Aβ25–35 for 30 min before beginning the detection of ESR spectra. Cell suspensions (i.e., 5×10^6 cells/ml) in the culture medium containing 50 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, Tokyo, Japan). The instrument conditions were set as follows: magnetic field = 336.85 ± 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.

Spin trapping in the 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 instrument conditions were set as follows: magnetic field = 336.85 ± 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.

Statistics

Values are expressed as the means ± SE. All parameters were analyzed by a one-way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test for specific comparisons. The significance was established at a level of $P < 0.05$.

Results

Confirmation of microglial phagocytosis of PS/PC liposomes

First, in order to confirm that the prepared 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, the microglia were fixed with 4% PFA and examined by fluorescence microscopy. The fluorescent images were merged with the corresponding phase-contrast images. As shown in Fig. 1A, well-defined microglial cytoplasm was observed to fill with fluorescently labeled PS/PC liposomes (green). In contrast, few PC liposomes labeled with the fluorescence were observed in the microglial cytoplasm (Fig. 1B). These findings indicate that PS/PC liposomes, but not PC liposomes, were phagocytosed by microglia.

Effect of PS/PC liposomes on the TNF-α production by Aβ/IFN-γ-activated microglia

We next investigated the effect of PS/PC liposomes on the TNF-α production by Aβ/IFN-γ-activated microglia. The incubation of primary cultured rat microglia with 50 μM Aβ25–35 combined with 100 U/ml IFN-γ for 24 h resulted in significant increases in the accumulation of TNF-α, whereas neither Aβ25–35 alone nor IFN-γ alone were able to activate the microglia to release TNF-α (Fig. 2A). The massive increase was significantly reduced by the pretreatment with PS/PC liposomes for 1 h in a dose-dependent manner (Fig. 2A).

In line with the results on the protein levels, the suppressive effect of PS/PC liposomes on the expression of mRNA

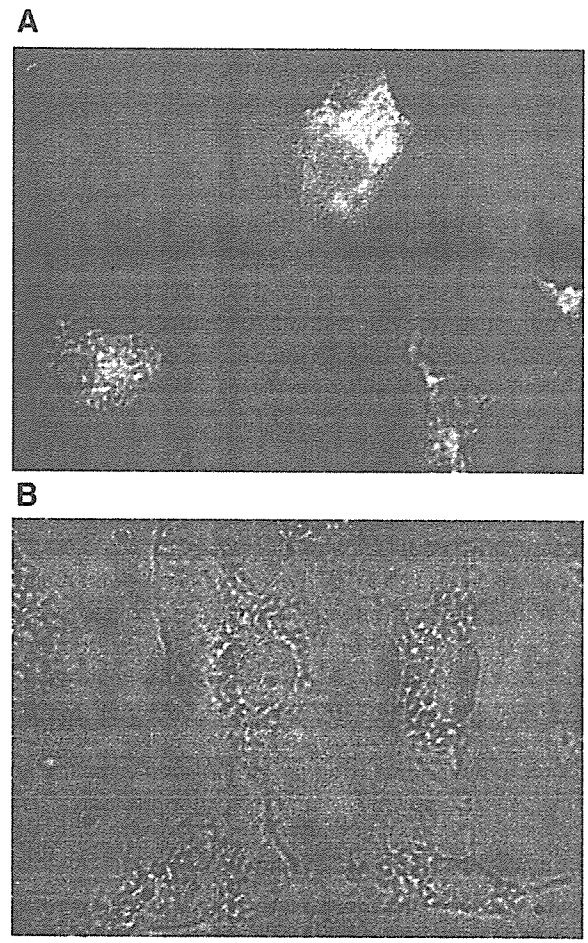

Fig. 1. Microglial phagocytosis of PS/PC liposomes. A typical fluorescence microphotograph showing phagocytosis of NBD-labeled PS/PC liposomes (green) by primary cultured rat microglia. The fluorescent image was merged with the corresponding phase-contrast image.

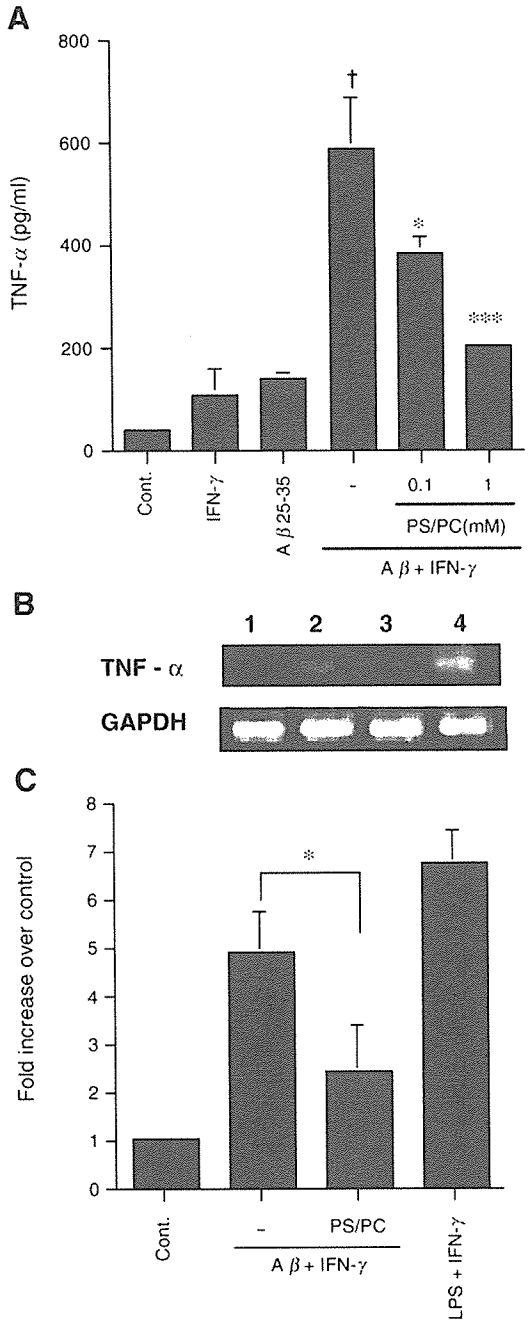

Fig. 2. Effect of PS/PC liposomes on the TNF-α production by Aβ/IFN-γ-activated microglia. (A) Primary cultured rat microglia were incubated with 50 μM Aβ25–35 and/or 100 U/ml IFN-γ at 37°C with or without pretreatment of PS/PC liposomes for 1 h. After 24 h, the collected media were assayed for TNF-α accumulation using ELISA. Data are the mean values ± SE (n=3). †P<0.0001, compared with control. *P<0.05, ***P<0.001, compared with Aβ25–35 + IFN-γ. (B) A representative RT-PCR analysis of the expression of mRNA encoding TNF-α in primary cultured rat microglia incubated with stimuli for 4 h with or without pretreatment of 1 mM PS/PC liposomes for 1 h. The administration of 10 ng/ml LPS combined with 100 U/ml IFN-γ was conducted as a positive control for the expression of TNF-α mRNA. Lane 1, control (medium); 2, Aβ25–35 + IFN-γ; 3, Aβ25–35 + IFN-γ + PS/PC liposomes; 4, LPS + IFN-γ. (C) Individual TNF-α mRNA levels were normalized to the corresponding levels of the mRNA encoding GAPDH. The results are expressed as the fold increase in the ratio of treated cell groups over the control. Data are the mean values ± SE (n = 3). *P < 0.05.

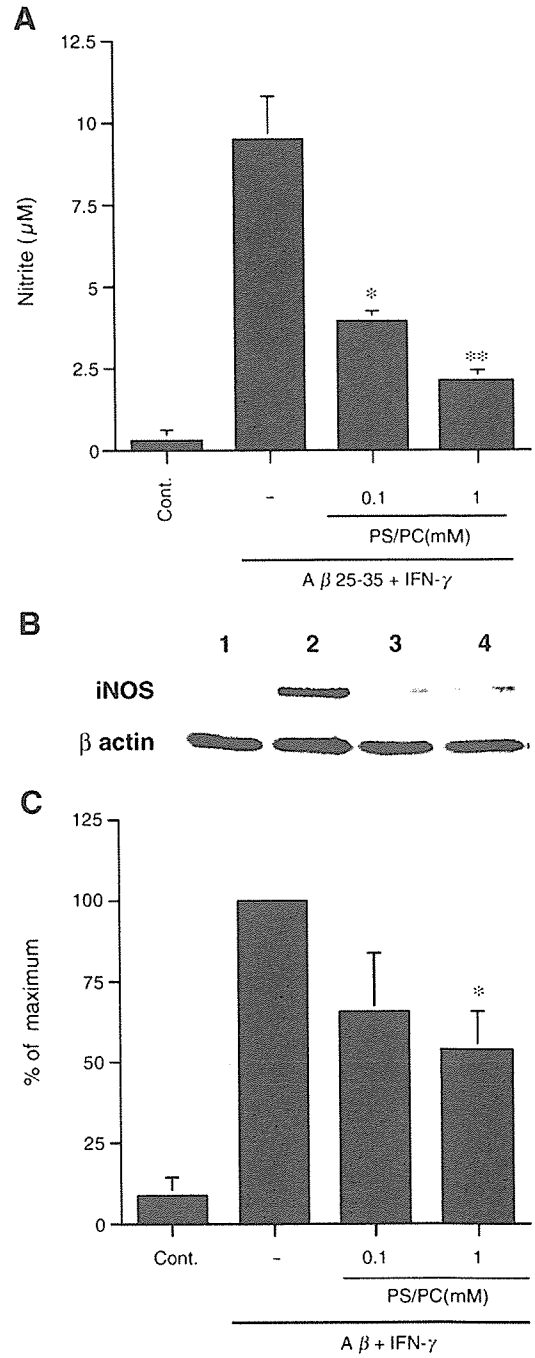

Fig. 3. Effect of PS/PC liposomes on the NO production by Aβ/IFN-γ-activated microglia. (A) Primary cultured rat microglial cells were incubated with 50 μM Aβ25–35 and 100 U/ml IFN-γ at 37°C with or without pretreatment of 1 mM PS/PC liposomes for 1 h. After 72 h, the collected media were assayed for NO accumulation using the Griess reaction. Data are the mean values ± SE. (n = 3). *P < 0.05, **P < 0.01, compared with Aβ25–35 + IFN-γ. (B) Representative Western blotting analysis of the expression of iNOS in 6-3 microglial cells incubated with Aβ25–35 + IFN-γ for 12 h with or without pretreatment of PS/PC liposomes for 1 h. Lane 1, control (medium); 2, Aβ25–35 + IFN-γ, 3, Aβ25–35 + IFN-γ + PS/PC liposomes (0.1 mM); 4, Aβ25–35 + IFN-γ + PS/PC liposomes (1 mM). (C) Individual iNOS expression levels were normalized to the corresponding levels of β-actin. The results are expressed as the percentage of the maximum levels (i.e., Aβ25–35 + IFN-γ). Data are the mean values ± SE. (n = 3). *P < 0.05 compared with Aβ25–35 + IFN-γ.