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Effects of plasmalogens on systemic lipopolysaccharide-induced glial activation and β-amyloid accumulation in adult mice

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Neuroinflammation essentially involves an activation of glial cells as the cause/effect of neurodegenerative diseases such as Alzheimer's disease (AD). Plasmalogens (Pls) are glycerophospholipids constituting cellular membranes and play significant roles in membrane fluidity and cellular processes like vesicular fusion and signal transduction. Intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 250 μg/kg) for 7 days resulted in the morphological changes and increase in number of Iba-1$^+$ microglia showing neuroinflammation in the adult mouse hippocampus. The LPS-induced activation of glial cells was significantly attenuated by i.p. pretreatment with Pls dissolved in corn oil. In addition, systemic injection of LPS induced Aβ_{1-16}^+ neurons in the hippocampus were also abolished by application of Pls. Finally, contents of Pls in the hippocampus decreased after LPS injection, and the reduction was suppressed by administration of Pls. These findings suggest an antiamyloidogenic effect of Pls, implicating a possible therapeutic application of Pls against AD.

Keywords: neuroinflammation; phospholipids; microglia; Alzheimer's disease

Introduction

It has been shown that systemic administration of lipopolysaccharide (LPS), a ligand for Toll-like receptor (TLR) 4, induces an impairment of cognitive behavior in mice.[1–3] Lee *et al.*[2] have reported that the production of β-amyloid protein (Aβ) and the activity of β-secretase, a key rate-limiting enzyme that initiates Aβ formation in the cortex and hippocampus increased in adult, but not young mice, following a single intraperitoneal (i.p.) injection of LPS. Furthermore, an intracellular accumulation of Aβ in the pyramidal neurons of the hippocampus was immunohistochemically demonstrated after daily LPS injections for 7 days.[2] Although the precise mechanisms for the LPS-induced amyloidogenesis have not yet been determined, it is likely that neuroinflammation, which is characterized by activation of glial cells and increased expression of cytokines, chemokines, and reactive oxygen/nitrogen species (ROS/RNS), plays significant roles, since glial cell–mediated inflammatory responses induce Aβ deposition in the brain.[4–6]

Plasmalogens (Pls) are unique glycerophospholipids that contain a vinyl ether bond at the *sn*-1 position of the glycerol moiety. They are found in all mammalian tissues, especially in heart and brain as ethanolamine Pls (PlsEtn), which are much more abundant than choline Pls (PlsCho).[7] Pls release either docosahexaenoic acid (DHA) or arachidonic acid (ARA) from the *sn*-2 position through the activation of Pls-selective phospholipase A_2 (Pls-PLA_2).[8,9] Pls are not only structural membrane components and reservoirs for second messengers,

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but they are also involved in membrane fusion, ion transport, and cholesterol efflux.[7] In addition, the vinyl ether bond at the *sn*-1 position makes Pls more susceptible to oxidative stress than the corresponding ester-bonded glycerophospholipids, thereby acting as antioxidants and protecting cells from oxidative stress.[10–13]

It has been shown that patients suffering from Alzheimer's disease (AD) have reduced PlsEtn levels in the cortex and hippocampus.[14–16] The reduction of PlsEtn seems to be specific since other neurodegenerative diseases, such as Huntington's and Parkinson's disease, do not show the decrease in the corresponding affected brain regions (caudate nucleus and substantia nigra, respectively).[7,14,17] Furthermore, circulating PlsEtn levels are also decreased depending on the severity of dementia.[18,19] Therefore, it is possible that Pls are involved in the pathology of AD. In this study, we focused on the LPS-induced amyloidogenesis and sought to elucidate (1) the effects of plasmalogen on neuroinflammation and Aβ accumulation in the hippocampus, and (2) changes in hippocampal plasmalogen content following peripheral administration of LPS in adult mice.

Materials and methods

All of the experimental procedures involving the use of animals were approved by the Ethics Committee on Animal Experiments, Kyushu University, and were in accordance with the Guiding Principles for the Care and Use of Animals of the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used for the studies.

Animals

Male C57/6J mice weighing 25–30 g (10 months old) were used in all experiments. Animals were housed in three cages (five per cage) at a temperature of 22 ± 2 °C with 12 h light/12 h dark cycle (light on at 8:00) and had free access to laboratory food and water. The mice were randomly divided into three groups: control, LPS, and LPS + Pls groups. LPS (Sigma-Aldrich, St. Louis, MO) was dissolved in saline, while Pls were in corn oil and then sonicated to ensure complete solubilization. The LPS group received intraperitoneal (i.p.) injection of LPS (250 μg/kg) followed by vehicle for Pls, corn oil, in the morning (9:00–10:00) daily for 7 days before sacrifice. The LPS + Pls group was treated with LPS and Pls (20 mg/kg), while the control group was given saline and corn oil for 7 days.

Plasmalogen preparation

Pls used in this study were prepared from chicken breast muscle by the same method as reported previously.[20] A high-performance liquid chromatography (HPLC) for phospholipids separation[21] indicated that the purified Pls consisted of 47.6% PlsEtn, 49.3% PlsCho, 2.4% sphingomyelin (SM), and 0.5% other phospholipids.

Immunohistochemistry and immunofluorescence

Mice were deeply anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The brain was removed, post-fixed for 24 h, and transferred successively to 20% and 30% sucrose solutions. Subsequently, brains were frozen on a cold stage and sliced into 30 μm in thickness using cryostat. The sections were permeabilized with 0.3% Triton-X 100 (Sigma-Aldrich, St. Louis, MO) in PBS for 15 min and blocked in PBS containing 1% BSA and 5% normal donkey serum (Jackson ImmunoResearch Lab., West Grove, PA) for 60 min at room temperature. Sections were incubated in the blocking solution (Block Ace, Dainippon Pharmaceutical, Japan) for 30 min at room temperature, and then incubated with rabbit polyclonal antibody against Iba-1 (1:10000; Wako Pure Chem. Indus., Osaka, Japan), which is known to have a specific affinity to the microglial Ca^{2+}-binding protein, and is highly expressed by activated microglia in 10% Block Ace in PBS, at 4 °C overnight. Other sections were incubated with antibody against $A\beta_{1–16}$ (1:1000; Abcam, Cambridge, UK) and NeuN (1:1000; Millipore, Billerica, MA). The rinsed sections were incubated for 6 h with Alexa Fluor 488 goat antirabbit IgG or Alexa Fluor 568 goat antimouse IgG (1:1000; Invitrogen, Eugene, OR) at room temperature. Every treatment was followed by washing three times for 5 min with PBS. Sections were then mounted in the perma fluor aqueous mounting medium (Thermo Fisher Scientific, Waltham, MA).

Quantitative analysis of fluorescence intensity

All samples were analyzed with a confocal laser-scanning microscope (LSM510 Meta; Carl Zeiss, Germany). The number of glial cells in 60–80 areas of 200 μm $\times$ 200 μm in five slices per brain was counted and the averaged number/4 $\times$ 10^4 μm^2 for each brain was obtained.

Measurement of plasmalogen contents in the hippocampus and PFC

Mice were deeply anesthetized with pentobarbital and (50 mg/kg) and transcardially perfused with sterile PBS. The brain was removed and hippocampus was dissected in a dish filled with ice-cold PBS. The samples (300–500 mg) were stored at -80 °C until plasmalogen measurement. The total lipids was extracted by the method of Folch *et al.*,[22] and relative composition of phospholipid classes, including Pls, was measured as reported previously.[21]

Statistical analysis

The results are expressed as the mean $\pm$ SEM. The number of Iba-1^+ cells were compared with one-way analysis of variance (ANOVA) followed by a *post hoc* (Scheffe's) test. Changes in PlsEtn contents and the ratio of PlsEtn/Phosphatidyl Etn (PEtn) after LPS and Pls injection were evaluated by the nonparametric Kruskal–Wallis test, which was followed by the Steel test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

Suppression of glial activation by Pls

As shown in Figure 1A-a, the control group that received saline and corn oil for 7 days showed

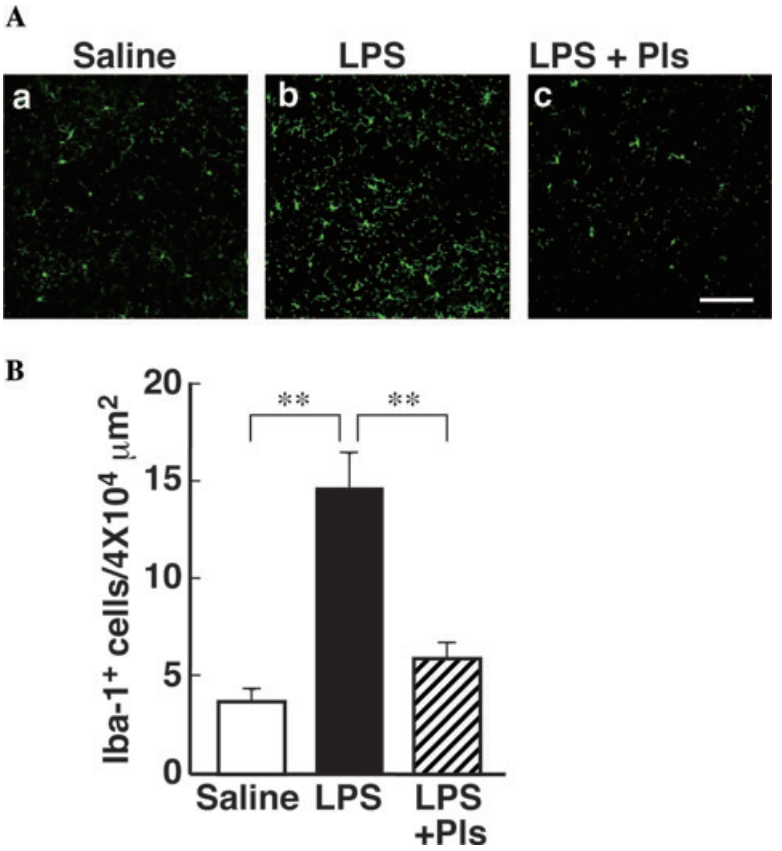

Figure 1. **Activation of glial cells following LPS (i.p.) and suppression by simultaneous application of Pls in the mouse hippocampus. (A) Number of Iba-1–positive microglia and intensity of immunoreactivity (a, green) increased with LPS treatment (b), which was suppressed by simultaneous application of Pls (c). Scale bar, 100 μm. (B) Summary of the LPS-induced increases in number of microglia, and suppression by Pls (each bar, n = 8). $^{**}P < 0.01$.**

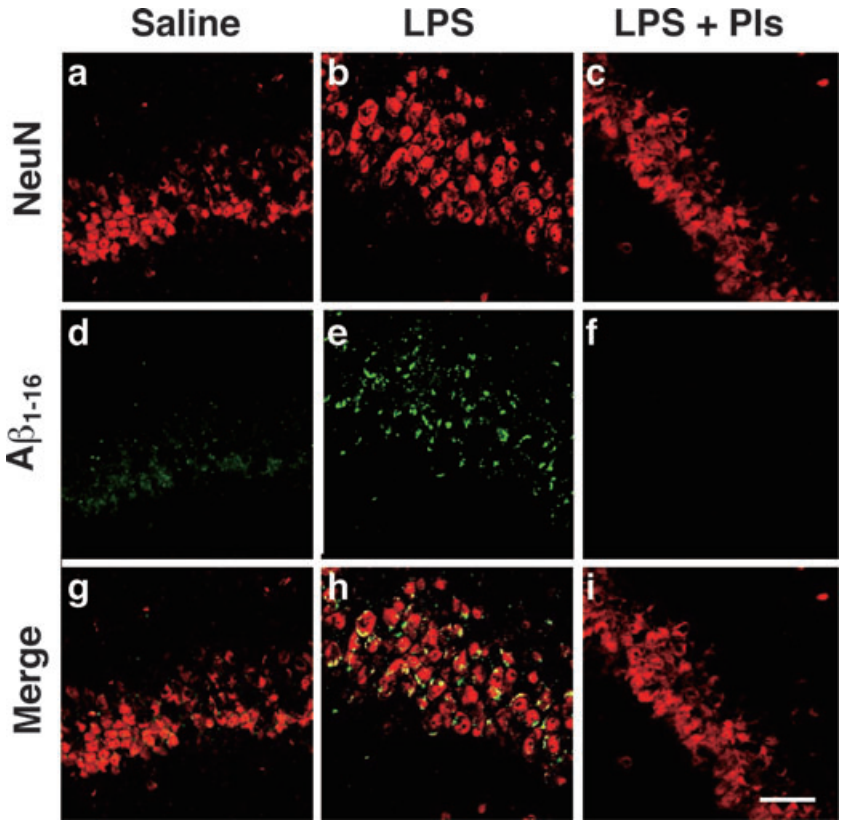

Figure 2. Accumulation of the Aβ protein following LPS (i.p.) and suppression by simultaneous application of Pls in the mouse CA1 region of the hippocampus. Neurons were stained with NeuN (red, a–c). A slight fluorescence for $A\beta_{1-16}$ immunoreactivity (green) of the control group (d) increased following LPS treatment (e), which completely abolished by Pls (f). $A\beta_{1-16}$ fluorescence merged with NeuN immunoreactivity, indicating an intracellular localization of Aβ (g and h). Scale bar, 50 μm.

typical features of Iba-1–positive (green) resting microglia with a small and compact soma bearing ramified processes in the hippocampus. However, itraperitoneal administration of LPS (250 μg/kg/day) for 7 days (LPS group) resulted in neuroinflammation, with an increased number of microglia and intense immunoreactivity and the activated phenotype of cellular hypertrophy and retraction of cytoplasmic processes (Fig. 1A-b). As shown in Figure 1A-a, the increase in activated microglia was suppressed by simultaneous administration of Pls (20 mg/kg) (LPS + Pls group). Figure 1B shows the summary of the LPS-induced increase in the number of glial cells and suppression by Pls (each bar, $n = 8$). One-way ANOVA indicated significant differences in the number of microglia (Fig. 1B; $F(2,21) = 44.7$, $P < 0.01$) among groups. The *post hoc* test indicated that the LPS group was different from the control and LPS + Pls groups (Scheffe's test, $P < 0.01$), but the LPS + Pls group was not different from control.

Suppression of LPS-induced Aβ accumulation by Pls

As shown in Figure 2, a slight fluorescence for $A\beta_{1-16}$ immunoreactivity (green) in the CA1 pyramidal cells of the hippocampus in the control group (Fig. 2d) was apparently increased in the LPS group (Fig. 2e), which was completely abolished in the LPS + Pls group (Fig. 2f). Pyramidal neurons were stained with NeuN (red, Figs. 2a–c), and most of $A\beta_{1-16}$ fluorescence merged with NeuN immunoreactivity, indicating an intracellular localization of Aβ (yellow in Fig. 2h).

Changes in plasmalogen contents in the brain after LPS and Pls treatment

As shown in Table 1, PlsEtn was much higher than PlsCho levels in the hippocampus. Relative content

Table 1. Changes in phospholipid content in the hippocampus after LPS and Pls injection

	Control	LPS	LPS + Pls
PlsEtn	23.98 ± 0.80	$20.49 \pm 0.44^{*}$	22.28 ± 0.70
PEtn	19.98 ± 0.47	21.87 ± 0.76	20.41 ± 0.51
PlsCho	0.51 ± 0.09	0.40 ± 0.03	0.47 ± 0.05
PCho	36.70 ± 1.63	39.19 ± 0.53	37.84 ± 1.43
SM	5.44 ± 0.64	5.16 ± 0.44	5.51 ± 0.68
PS	11.93 ± 0.79	11.14 ± 0.44	11.91 ± 0.710
PI	1.65 ± 0.12	1.83 ± 0.08	1.74 ± 0.07

PlsEtn, ethanolamine plasmalogen; PEtn, phosphatidyl ethanolamine; PlsCho, choline plasmalogen; PCho, phosphatidyl choline; SM, sphingomyelin; PS, phosphatidyl serine; PI, phosphatidyl inositol. Values are the means ± SEM ($n = 5$) of % contents of phospholipids, except for the last row. $^{*}P < 0.05$ compared with the control group (Steel test for multiple comparisons).

of PlsEtn was significantly decreased by LPS injection (Kruskal–Wallis test, $\chi^2(2) = 7.74$, $P < 0.05$). Multiple comparison analysis by the Steel test revealed that PlsEtn content in the LPS group, but not in LPS + Pls, significantly decreased compared with the control group ($P < 0.05$, each group, $n = 5$). Contents of other phospholipids showed no significant changes following any treatments.

Discussion

One of the possible mechanisms of the LPS-induced amyloidogenesis that has been recently suggested is that the activity of β- and γ-secretase, which are deeply involved in the amyloidogenic processing from amyloid precursor protein,[23] increases in the cortex and hippocampus following systemic injection of LPS.[2] It has been shown that proinflammatory cytokines, as well as ROS/RNS, released from activated microglia augment Aβ formation through upregulating β-secretase mRNA and enzymatic activity.[5,24] Microglia are activated further through receptors for advanced glycation end product (RAGE), which bind Aβ, or induce phagocytosis of Aβ, thereby amplifying generation of ROS/RNS and cytokines.[23]

In addition to the activation of glial cells and Aβ accumulation, here we showed for the first time that plasmalogen contents in the hippocampus deceased following LPS administration (Table 1). A possible mechanism of the decrease in Pls during neuroinflammation may result from an antioxidant property of Pls that protects cells from oxidative stress. It has been shown that Pls-specific vinyl ether bond at the *sn*-1 of the glycerol backbone is targeted by a vast variety of oxidants, including ROS/RNS,[10,11,13] and oxidative stress preferentially oxidizes PlsEtn over phosphatidyl ethanolamine,[25,26] resulting in the disruption of vesicular fusion in the synaptosomes and acetylcholine release.[27] This may at least partly explain why AD patients show a decrease in Pls content in the brain.[14–16] It has been suggested that an abnormal membrane lipid composition, namely, a decrease in the ratio of Pls to non-Pls ethanolamine glycerephospholipids, causes membrane instability in AD, which may contribute to amyloidogensis by cooperatively acting with amyloid cascade mechanism.[14] Furthermore, since PlsEtn are major endogenous lipid constituents that facilitate membrane fusion of synaptic vesicles associated with neurotransmitter release,[28,29] age-related and/or pathological alteration in Pls content is suggested to play important roles in neurological disorders including AD.[7] In fact, the decrease in Pls is shown to be closely correlated with the severity of dementia in humans.[18,19]

Several lines of evidence have suggested that there is a causal loop among neuroinflammation, Aβ accumulation, ROS/RNS production, and decrease in Pls. The LPS-induced activation of β-secretase,[2] which is predominantly localized in cholesterol-rich lipid raft,[30,31] causes accumulation of the Aβ protein. The Aβ-induced production of ROS/RNS enhances lipid peroxidation,[32,33] resulting in the decrease in Pls content, as mentioned previously. In addition, it has been shown that increased Aβ and ROS/RNS reduce expression of alkyl-dihydroxyacetonephosphate-synthase, a rate-limiting enzyme for plasmalogen *de novo* synthesis, due to the dysfunction of peroxisomes where Pls are biosynthesized, resulting in the decrease in plasmalogen level.[34] It is also reported that the LPS-induced neuroinflammation itself can cause the loss of Pls by inducing TNF-α that downregulates another key enzyme in plasmalogen biosynthesis in peroxisomes, glycerol-3-phosphate-*O*-acyltransferase,[35] and by upregulating myeloperoxidase that generates one of the reactive species, hypochlorous acid (HOCl), in the brain, targeting Pls to be oxidized.[36] Finally, Pls-PLA_2, which degradates Pls to release DHA or ARA from the *sn*-2

position of the glycerol moiety, is possibly activated by ceramide produced under inflammatory conditions and contributes to the loss of Pls in the brain.[9,37]

The reduction of Pls has been shown to induce a decreased ability to intracellular cholesterol transport from cell membrane to the endoplasmic reticulum, resulting in an increase in cholesterol on the cell surface.[38] On the other hand, it is well known that the generation and clearance of Aβ are affected by cholesterol metabolism, which is evidenced by the identification of a variant gene of the apolipoprotein E, a cholesterol transporter, as a major genetic risk factor for AD.[23,39,40] Depletion of cholesterol inhibits the generation of Aβ,[41,42] while an increase in cholesterol promotes secretion of Aβ.[39,40,43] Thus, a vicious circle of LPS-induced Aβ accumulation that decreases the Pls level, which leads to an increase in cholesterol, and thereby enhances further generation of Aβ, is established under the pathological condition of neuroinflammation.

In this study, we showed that the LPS-induced activation of glial cells (Fig. 1), accumulation of the Aβ protein (Fig. 2), and decrease in PlsEtn (Table 1) in the hippocampus were suppressed by peripheral injection of Pls. The precise mechanism of Pl effects is not known in this study. Since glial activation, Aβ accumulation, and Pls reduction are all involved in the vicious circle mentioned previously, it is possible that effective supplementation of Pls can improve all of these pathological disorders. The most important question may be whether peripheral Pls can enter the brain. There are no reports thus far that Pls directly cross the blood–brain barrier (BBB). Therefore, it cannot be excluded that the antioxidative effects of Pls are exerted outside the brain to suppress the primary inflammation induced by peripheral LPS. However, it has been shown that Pls in the serum decrease in parallel with, or even earlier than, the decrease in brain Pls in AD patients.[18,19] Furthermore, our results showed that the LPS-induced decrease in plasmalogen contents in the hippocampus was corrected by the peripheral administration of Pls (Table 1). Therefore, it is possible that a peripheral supplementation of Pls could be expected to have an effect on the central nervous system (CNS).

Another question is whether the effective molecules in our experiment are not Pls themselves, but polyunsaturated fatty acids (PUFAs), which Pls must carry at the *sn*-2 position. There are several lines of evidence showing that *n*-3 PUFAs, such as eicosapentaenoic acid, DHA, and its derivative, neuroprotectin D1, have anti-inflammatory and neuroprotective effects.[44–47] Furthermore, DHA has been reported to suppress production of the Aβ protein through multiple mechanisms including inhibition of β-/γ-secretase activities and alteration of membrane cholesterol distribution.[48–50] Since the purified Pls used in this study contain DHA and its precursor, α-linolenic acid, especially in PlsEtn, it is possible that DHA derived from PlsEtn plays a significant role in the CNS effects of Pls. In fact, it has been shown that DHA is synthesized from α-linolenic acid and incorporated into phospholipids in the liver, and is then transported to the brain through the peripheral circulation.[51] On the other hand, it has been shown that lyso-type phospholipids, which contain DHA at the *sn*-2, show preferential transfer through an *in vitro* model of the BBB over DHA.[52] This finding suggests that Pls-containing DHA are more effective to exert the CNS actions than DHA itself.

This study suggests that peripheral administration of Pls, of which extraction techniques have been recently developed,[20,21] may suppress neuroinflammation in the brain. Although further investigations concerning mechanisms of the CNS effects are needed, including the pathways for entering into the brain, the present results indicate the possibility of Pls as a new preventive/therapeutic strategy for Alzheimer's disease.

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Conflicts of interest

The authors declare no conflicts of interest.

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