

Anti-neuroinflammatory potential of porcine liver decomposition products in improving behavioral abnormalities: Effects on formalin- and LPS-induced inflammation

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Abstract. Porcine liver decomposition product (PLDP) is a functional food that enhances delayed memory recall and frontal lobe function in humans and exerts antidepressant and anxiolytic effects. In the present study, the previously unexplored anti-inflammatory effects of PLDP were investigated both *in vitro* and *in vivo*. The effects of PLDP on interleukin (IL)-1 β production were examined in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells and on their transition to an M1 or M2 phenotype. The effects of PLDP administered 30 min before the initiation of a formalin-induced inflammation-related behavior were also evaluated. Furthermore, the effects of PLDP on LPS-induced inflammation related to cytokine production and behavior *in vivo* were examined. PLDP was orally administered for five consecutive days and 24 h after LPS administration the mice were evaluated in an open field test and plasma cytokine production was measured. PLDP and its lipid fraction, PLDP extracted lipids (PEL) suppressed LPS-induced IL-1 β production. Flow cytometry revealed that

PEL shifted Raw 264.7 cells to the M2 phenotype. Moreover, PEL reduced phase 2 inflammation-related behavior in formalin-induced inflammation. In the LPS-induced inflammation model, PLDP improved the LPS-attenuated locomotor activity and exploratory behavior and reduced the increased plasma interferon- γ levels. These results suggest that PLDP exerts anti-inflammatory effects and shifts macrophages to the M2 phenotype. Furthermore, our results indicate that the lipid components in PLDP, especially phospholipids, could exert considerable central neuroprotective effects.

Introduction

Porcine liver decomposition product (PLDP), obtained by treating porcine liver with proteases, is abundant in proteins, phospholipids, peptides, essential amino acids, B vitamins, and minerals such as iron and zinc (1). Matsuda *et al* (2) evaluated the efficacy of PLDP in improving cognitive function using the Hasegawa Dementia Scale-Revised (HDS-R) and revealed that it improves delayed memory recall and HDS-R scores. Additionally, Matsuda *et al* (3) conducted a randomized, double-blind, placebo-controlled study to examine the effects of PLDP on cognitive function using the Wechsler Memory Scale-Revised in healthy adults aged 40 years or older, revealing that PLDP improves visual memory and delayed recall ability. Furthermore, Suzuki *et al* (4) investigated changes in the total HDS-R score and individual HDS-R question items following oral PLDP administration in participants with HDS-R scores between 15 and 23 points and confirmed that PLDP improves delayed verbal memory recall and verbal fluency task. Verbal fluency tasks reflect the function of the prefrontal cortex (5), which has been linked to depressive symptoms (6). Therefore, PLDP may improve prefrontal cortex function and exert antidepressant and anti-anxiety effects.

Phosphatidylcholine and lysophosphatidylcholine present in PLDP were previously investigated and a radial arm maze experiment using rats was conducted to examine memory acquisition when choline, a breakdown product of PLDP, was

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Abbreviations: BDNF, brain-derived neurotrophic factor; COVID-19, coronavirus disease 2019; HDS-R, Hasegawa dementia scale-revised; IFN, interferon; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; OFT, open field test; PEL, PLDP extracted lipids; PLDP, porcine liver decomposition product; SARS CoV 2, severe acute respiratory syndrome coronavirus 2; TLR, toll-like receptor; TNF, tumor necrosis factor

Key words: PLDP, behavioral changes, neuroinflammation, PEL

administered (7). It was confirmed that the administration of choline promotes both the memory acquisition process and delayed recall. In the aforementioned study, the levels of brain-derived neurotrophic factor (BDNF) in the hippocampus were measured and it was confirmed that the production of BDNF increases at both the gene and protein levels in rats with choline administration (7).

Tsukahara *et al* (8-10) reported that PLDP suppresses the production of inflammatory cytokines, suggesting that the underlying mechanism could be a shift from M1 to M2 microglial phenotype induced by PLDP extracted lipids (PEL), the phospholipid fraction contained in PLDP. Microglia play a crucial role in the inflammatory response of the central nervous system in neurodegenerative diseases. Excessive microglial activation and inflammatory cytokine release can cause neuronal damage and cognitive decline with signaling pathways such as the NF- κ B and MAP kinase pathways believed to play an important role in the molecular mechanisms underlying microglial neurotoxicity (11,12).

Recent research has revealed that this neuroimmune response, called neuroinflammation, is an important precursor to various neurological diseases. Therefore, it is considered that balancing the induction and inhibition of neuroinflammation by PLDP is important for alleviating brain diseases. Furthermore, PLDP, which has been shown to improve learning and memory and regulate frontal lobe function, is expected to improve symptoms caused by neuroinflammation. In the present study, the anti-inflammatory effects of PLDP and PEL were first investigated *in vitro*. Further, the anti-neuroinflammatory efficacy of PLDP was investigated *in vivo* using formalin-induced inflammation-related behavior and lipopolysaccharide (LPS)-induced inflammation-related cytokine production and behavior as indicators.

Materials and methods

PLDP and preparation of PEL. PLDP (cat. no. 170130) was supplied by Sugar Lady Cosmetics Co., Ltd. The components of PLDP are listed in Table I. PEL was extracted from PLDP using the Bligh & Dyer method (13). Briefly, PLDP was mixed with equal amounts of 0.2 M acetic acid, followed by the addition of 5-fold the volume of methanol and 2.5-fold the volume of chloroform. The mixture was stirred for 1-2 min using a vortex. After allowing it to stand for 10 min, 5 ml of chloroform and distilled water were added to the mixture and stirred for 1 min. The mixture was centrifuged at 1,500 $\times$ g for 10 min and the lower layer was collected. An equal amount of washing solution (water: methanol: chloroform=47: 48: 3) was added to the collected sample, and the mixture was centrifuged at 1,500 $\times$ g for 10 min. The lower chloroform layer was collected and dried using a nitrogen gas evaporator.

Inhibition of interleukin (IL)-1 β production. LPS (0000130083; *E. coli*, O111:B4) was purchased from Sigma-Aldrich. The enzyme-linked immunosorbent assay (ELISA) kits used for detection of mouse IL-1 β were purchased from Abcam. Murine macrophage Raw 264.7 cells (RIKEN BRC) were seeded in a 24-well plate (5 $\times$ 10⁵ cells/well) and cultured (37°C, 5% CO₂) for 24 h. LPS, LPS + PLDP, or LPS + PEL were then added and incubated for an additional

48 h. LPS was added at a concentration of 100 ng/ml, PLDP was administered at a 1,000-fold dilution of the original solution, and PEL was administered at 25 μ g/ml. After the final incubation, IL-1 β levels in the supernatant were measured as an indicator of neurotoxic factors.

Effect of PEL on M1 and M2 macrophages. Antibodies used for flow cytometry included monoclonal antibodies CD86 (1:100; cat. no. 11-0862-81) and CD16/CD32 (1:100; cat. no. 14-0161-82), both purchased from Abcam; and CD206 (1:100; cat. no. 141705), purchased from BioLegend, Inc. Raw 264.7 cells (1 $\times$ 10⁶ cells) were treated for 48 h with PEL (25 μ g/ml) + LPS (100 ng/ml) or only LPS (100 ng/ml) and then immuno-stained at 4°C for overnight for flow cytometric analysis. Briefly, the cells were collected, and the Fc receptors were blocked for 20 min on ice using the CD16/CD32 monoclonal antibody. This was followed by incubation with the CD86 monoclonal antibody at room temperature to label the M1 macrophages. After washing, the CD206 monoclonal antibody was added for labeling M2 macrophages. Following the staining, the samples were evaluated using a BD FACSCalibur flow cytometer (BD Biosciences). The data was analyzed using the BD CellQuest Pro software (part no. 349226; Rev A July 2002; BD Biosciences).

Effect of PLDP on the formalin-induced inflammation model. Male ddY mice (n=14, Japan SLC, Inc.) weighing 20-26 g was used in this experiment. The mice were maintained in a controlled environment with a 12/12-h light-dark cycle (light period 07:00-19:00, dark period 19:00-7:00), a temperature of 23 $\pm$ 1°C, and humidity of 52 $\pm$ 2% until the experiment. The animals were allowed free access to solid food (SLC F2; CLEA Japan, Inc.) and tap water. The present study was planned and approved in accordance with the animal experiment regulations of Nihon Pharmaceutical University (approval no. AE2023-005; Saitama, Japan) and was performed using the fewest number of animals necessary and ensuring that pain was minimized.

Inflammation pain models, including the formalin-induced inflammation model, evaluate pain and analgesia by administering chemicals that stimulate peripheral nerves to induce inflammation and are broadly considered neuropathic pain models. In the present study, the formalin induced inflammation model was performed according to the method described by Tan-No *et al* (14). Prior to the experiment, mice were individually placed in transparent plastic cages (22.0 $\times$ 15.0 $\times$ 12.5 cm) to enable observation and allowed to acclimate to the environment for $\sim$ 1 h. PLDP or distilled water (Otsuka Pharmaceutical Factory) was orally administered (0.2 ml/mouse, n=7) 30 min before injection of 20 μ l of 2% formalin (Fujifilm Wako Pure Chemical Industries) into the hind footpad of the mice. The formalin was diluted with saline (Otsuka Pharmaceutical Factory). Behavioral observations were performed for 30 min after formalin administration. The antinociceptive effect of PLDP was evaluated based on observed pain-related behaviors (licking and biting).

Effect of PLDP on the LPS-induced inflammation model. In total, 8-week-old C57BL/6J male mice (CLEA Japan, Inc.) were used for the experiment (15). The mice were placed in a

Table I. PLDP component analysis.

PLDP component		Amount
Moisture ^a		72.90%
Crude fat ^b		3.09%
Phospholipid subtype ^c		
PC	Total phosphatidylcholine	21.716 mg
LPC	Total lysophosphatidylcholine	3.390 mg
PE	Total phosphatidylethanolamine	2.569 mg
PI	Total phosphatidylinositol	1.076 mg
SM	Total sphingomyelin	0.764 mg
PA	Total phosphatidic acid	0.648 mg
PS	Total phosphatidylserine	0.085 mg

^aNormal-pressure heating drying method (105°C, 3 h); ^bEther extraction method; ^cPhospholipids were extracted from PEL using the Bligh & Dyer method (13) and analyzed by Liquid chromatography-tandem mass spectrometry method. PLDP, porcine liver decomposition product.

room with a controlled temperature of 25±1°C (12-h light/dark cycle) and allowed to acclimate for 7 days. After acclimatization, the mice were divided based on uniform body weight into three experimental groups (n=8/group) and housed in cages (345x403x177 mm). Water was provided ad libitum. This study was planned and approved in accordance with the animal experiment regulations of; University (approval no. AE2024-017; Saitama, Japan). The present study was conducted at the Kyoto Institute of Nutritional Pathology with the approval of the Animal Ethics Committee of the Kyoto Institute of Nutritional Pathology (approval date: May 7, 2024, approval number: 23027NY②; Kyoto, Japan). The experiment was also conducted with the minimum number of animals necessary, with care taken to minimize pain.

The experimental groups included group 1 (saline), group 2 (LPS), and group 3 (LPS + PLDP). From days 2 to 7 of the experiment, group 3 received orally administered PLDP [0.2 ml/20 g body weight (BW)] while groups 1 and 2 were administered an equivalent volume of saline. The administration periods of PLDP and saline were set based on the papers by Wakasugi *et al* (16) and Hong *et al* (17). On the 7th day of the experiment, LPS (*E. coli* O111:B4) adjusted to 0.1 mg/ml with saline was intraperitoneally administered to groups 2 and 3 at 0.1 ml/20 g BW. Group 1 administered an equal volume of saline only.

All animals were autopsied 8 days after the start of the experiment. After anesthesia using of sodium medetomidine (0.3 mg/kg B.W., Meiji Seika Kaisha, Ltd.), midazolam (4.0 mg/kg B.W.), and butorphanol tartrate (5.0 mg/kg B.W., Meiji Seika Kaisha, Ltd.) (15,18) with an intraperitoneal injection, and blood was collected from the abdominal vena cava. The blood was heparinized, and plasma was promptly separated, frozen, and stored at -80°C until use.

Open field test (OFT). Mice from each experimental group were individually placed in an open field (500x500x400 mm,

Obara Medical Industry Co., Ltd.), which was disinfected with alcohol and lit under 100 lux lighting conditions. The amount of spontaneous movement and time spent in the field center were analyzed for 10 min for each mouse. The OFT was conducted at 10:00 a.m.

Quantification of blood cytokine levels. The plasma concentrations of inflammatory cytokines, namely IL-6, IL-10, IL-12 p70, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1, and TNF, were measured using a cytometric bead array (Mouse Inflammation CBA kit; BD Biosciences), according to the manufacturer's protocol.

Statistical analysis. The data regarding the inhibition of IL-1β production were analyzed using one-way ANOVA, followed by multiple comparison analysis using the Tukey method to compare differences in the average values between the three groups. Results are presented as mean ± SE. After confirming the normality of the M1 and M2 macrophage flow cytometry data, the mean values between groups were compared using paired or unpaired t-tests, depending on whether the data were paired. After confirming the normality of the formalin-induced inflammation model data, the mean values between groups were compared using paired or unpaired t-tests, depending on whether the data were paired. Results are presented as the mean ± SE. Baseline data on total distance traveled, exploratory behavior, number of entries into the central area, time spent in the central area, and cytokine levels were established based on the data obtained from the first day of the OFT. Changes from baseline on the second day of the OFT were calculated. Comparisons were made between groups using the Holm multiple comparison test. For plasma cytokine concentrations, multiple comparison analysis was performed using the Tukey-Kramer method to compare the differences in the average values between the three groups. Moreover, statistical verification of the distribution of data was performed using the Smirnov rejection test. P<0.05 was considered to indicate a statistically significant difference for all statistical analyses.

Results

PLDP- and PEL-mediated inhibition of IL-1β production. IL-1β production induced by LPS stimulation was inhibited by ~95% when PLDP was diluted 1,000-fold from the original solution. Similarly, IL-1β production induced by LPS stimulation was inhibited by ~93% when PEL was used at a concentration of 25 μg/ml (Fig. 1).

Effects of PEL on M1 and M2 macrophages. The PEL-treated group exhibited an increased proportion of M2 macrophages compared with the control group (Fig. 2). Stimulation of Raw264.7 cells with LPS tended to shift the phenotype to M1, whereas addition of PLDP significantly shifted the phenotype to M2.

Effect of PLDP on the formalin-induced inflammation model. Pain and inflammation-related behaviors (licking and biting) were observed following the subcutaneous administration of 2% formalin (20 μl) into the hind footpads of mice. These behaviors included primary responses observed within 10 min

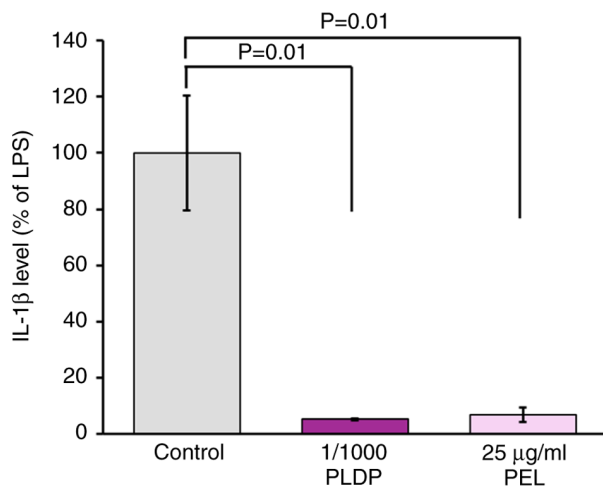


Figure 1. Effects of PLDP and its lipid fraction PEL on (IL)-1 β production in LPS-stimulated Raw 264.7 cells. IL-1 β production induced by LPS stimulation (100 ng/ml) was inhibited by ~95% when PLDP was diluted 1,000-fold from the original solution and by ~93% when PEL was used at a concentration of 25 μ g/ml. Data are expressed as the mean $\pm$ S.E (n=3/group); Cont=LPS; PLDP=PLDP + LPS (P=0.01); PEL=PEL + LPS (P=0.01). PLDP, porcine liver decomposition product; PEL, PLDP extracted lipids; IL, interleukin; LPS, lipopolysaccharide.

of subcutaneous administration and secondary responses observed between 10 and 30 min after administration. No significant differences in the primary responses were observed between the PLDP-administered and control groups; however, a significant reduction in pain-related behaviors was observed in the secondary response (Fig. 3).

Effect of PLDP on the LPS-induced inflammation model.

Significant changes in OFT behavior were observed on the first day after LPS administration, which gradually recovered and returned to baseline behavior. The percentage of change (mean $\pm$ SE) from day-one baseline in the mean total distance traveled, number of exploratory behaviors, number of central zone entries, and time spent in the central zone on the second day of the OFT, are shown in Fig. 4. The percentage change in the mean total distance traveled on the second day of the OFT was 76.3 $\pm$ 4.6% in group 1 (saline), 34.2 $\pm$ 2.4% in group 2 (LPS), and 43.2 $\pm$ 3.0% in group 3 (PLDP + LPS), with group 2 exhibiting the lowest value. Significant differences were observed between groups 1 and 2 and between groups 1 and 3, and a significant decrease in the total distance traveled (change rate) was observed due to LPS administration. Furthermore, a significant difference was observed between groups 2 and 3, indicating that PLDP administration significantly improved decrease in the total distance traveled (change rate) induced by LPS.

The rate of change (mean $\pm$ SE) in the number of exploratory behaviors on the second day of the OFT was 91.9 $\pm$ 10.4% in group 1, 26.8 $\pm$ 2.3% in group 2, and 37.0 $\pm$ 3.5% in group 3, with group 2 exhibiting the lowest value. A significant difference was observed between groups 1 and 2, indicating that LPS administration significantly reduced the number of exploratory behaviors. Furthermore, a significant difference was observed between groups 2 and 3, indicating that PLDP administration significantly mitigated the decrease in the number of exploratory behaviors (rate of change) induced by LPS.

The rate of change (mean $\pm$ SE) in the median number of entry attempts on the second day of the OFT was 50.2 $\pm$ 8.2% in group 1, 13.9 $\pm$ 2.7% in group 2, and 18.4 $\pm$ 2.1% in group 3, with group 2 exhibiting the lowest value. A significant difference was observed between groups 1 and 2, indicating that LPS administration significantly reduced the median number of entry attempts (rate of change). Conversely, no significant differences were observed between groups 2 and 3.

The rate of change (mean $\pm$ SE) in median dwell time on the second day of the OFT was 56.8 $\pm$ 11.7% in group 1, 15.5 $\pm$ 4.3% in group 2, and 24.0 $\pm$ 8.9% in group 3, with group 2 showing the lowest value. Significant differences were observed between groups 1 and 2, as well as between groups 1 and 3; however, no significant differences were observed between groups 2 and 3, with LPS administration leading to a significant decrease in median dwell time (rate of change).

Changes in plasma cytokine levels were analyzed using plasma collected on the eighth day after LPS administration; however, no significant changes were observed (P<0.05). In a separate experiment, plasma cytokine levels were also analyzed 24 h after LPS administration. The measured cytokine concentrations in abdominal vena cava blood plasma 24 h after LPS administration are depicted in Fig. 5.

The mean plasma concentration of IL-12p70 ($\pm$ SE) was 2.46 $\pm$ 1.31 pg/ml in group 1, 0.21 $\pm$ 0.21 pg/ml in group 2, and 0 pg/ml in group 3. Group 1 exhibited the highest value, followed by groups 2 and 3 respectively. No significant differences were observed between the groups.

The mean plasma TNF concentration was 5.95 $\pm$ 0.59 pg/ml in group 1, 39.93 $\pm$ 4.02 pg/ml in group 2, and 31.14 $\pm$ 1.92 pg/ml in group 3. Group 2 had the greatest value, and significant differences were observed between groups 1 and 2 and between groups 1 and 3. However, no significant differences were observed between groups 2 and 3.

Group 2 had the highest IFN- γ levels at 2.24 $\pm$ 0.08 pg/ml, while those in groups 1 and 3 were 1.32 $\pm$ 0.03 pg/ml and 1.86 $\pm$ 0.10 pg/ml, respectively. Significant differences were observed between all three groups (groups 1 and 2, 1 and 3, and 2 and 3).

Monocyte chemoattractant protein-1 (MCP-1) was highest in group 2 at 509.35 $\pm$ 100.98 pg/ml, while groups 1 and 3 had mean MCP-1 levels of 6.61 $\pm$ 2.19 and 442.73 $\pm$ 67.12 pg/ml, respectively. Significant differences were observed between groups 1 and 2 and between groups 1 and 3, but not between groups 2 and 3.

Similarly, group 2 had the highest IL-10 levels at 17.70 $\pm$ 0.77 pg/ml, while those in groups 1 and 3 were 2.68 $\pm$ 1.59 and 17.18 $\pm$ 0.93 pg/ml, respectively. Significant differences were observed between groups 1 and 2 and between groups 1 and 3; however, no significant differences were observed between groups 2 and 3.

IL-6 levels were highest in group 2 at 43.70 $\pm$ 6.17 pg/ml, followed by 2.84 $\pm$ 0.71 pg/ml in group 1 and 38.76 $\pm$ 5.49 pg/ml in group 3. Significant differences were observed between groups 1 and 2 and between groups 1 and 3, but not between groups 2 and 3.

Discussion

In the present study, it was demonstrated that PLDP, which has previously been shown to improve cognitive function in clinical

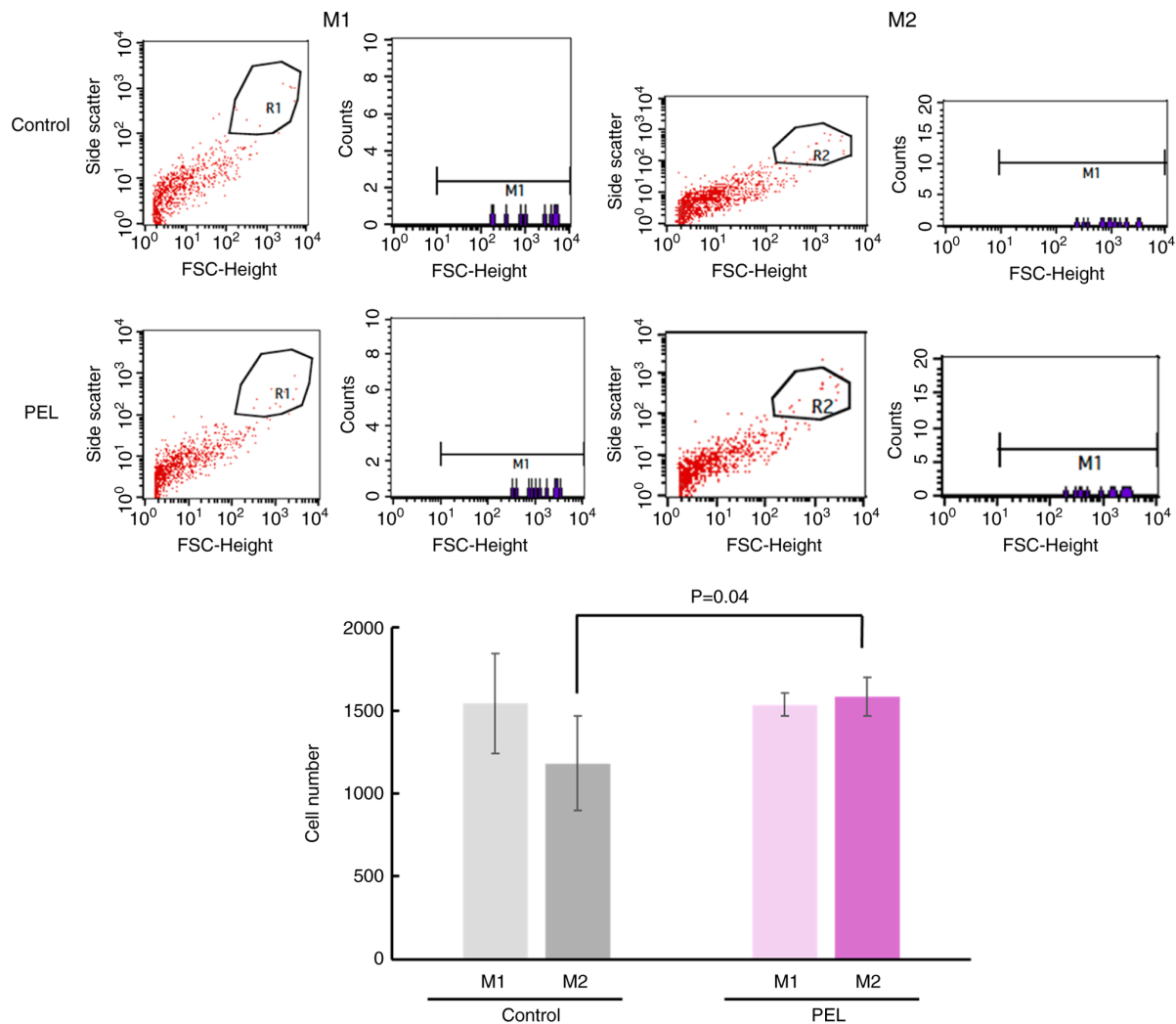


Figure 2. PEL activates macrophages and induces the M2 phenotype. Total phospholipids (PEL) were extracted from PLDP using the Bligh and Dyer method. Data are expressed as the mean $\pm$ SE (n=3); Control=LPS (100 ng/ml); PEL=PEL (25 μ g/ml) + LPS (100 ng/ml). PLDP, porcine liver decomposition product; PEL, PLDP extracted lipids; LPS, lipopolysaccharide.

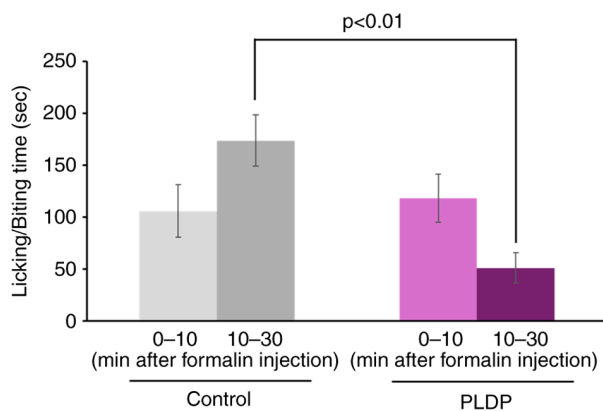


Figure 3. PLDP significantly reduces pain behaviors during the secondary reaction time in a formalin-induced acute pain model. PLDP was administered into the footpads of mice 30 min before the irritant formalin was administered. Data are expressed as the mean $\pm$ SE (n=7). PLDP, porcine liver decomposition product.

mechanism underlying this was attributed to a shift towards M2-type macrophages. These results suggest that PLDP may also exert an anti-inflammatory effect within the brain via M2 microglial cells. Similar activity was observed with PEL treatment, suggesting that the lipid fraction of PLDP may play a key role in the mitigation of chronic inflammation by PLDP. However, the *in vivo* effects of PLDP have been largely unexplored, primarily due to the lack of appropriate pathological models. The effects of PLDP were therefore investigated using a formalin-induced inflammation model, where IL-1 β plays a key role in the pathological mechanism. It was also found that a single administration of PLDP, 30 min before formalin administration, suppressed pain-related responses during the second phase. Furthermore, the effects of PLDP in an LPS-induced inflammation model were investigated and it was found that daily oral administration of PLDP for 5 days alleviated abnormal behavior and suppressed the increased IFN- γ levels induced by LPS administration.

Infections with viruses such as SARS CoV 2 or influenza can cause acute inflammation in humans, leading to the production of inflammatory cytokines such as IL-1 β and antiviral cytokines such as IFN in the periphery, which cause

trials, suppressed the production of IL-1 β in LPS-stimulated Raw 264.7 macrophages. Furthermore, it was revealed that the

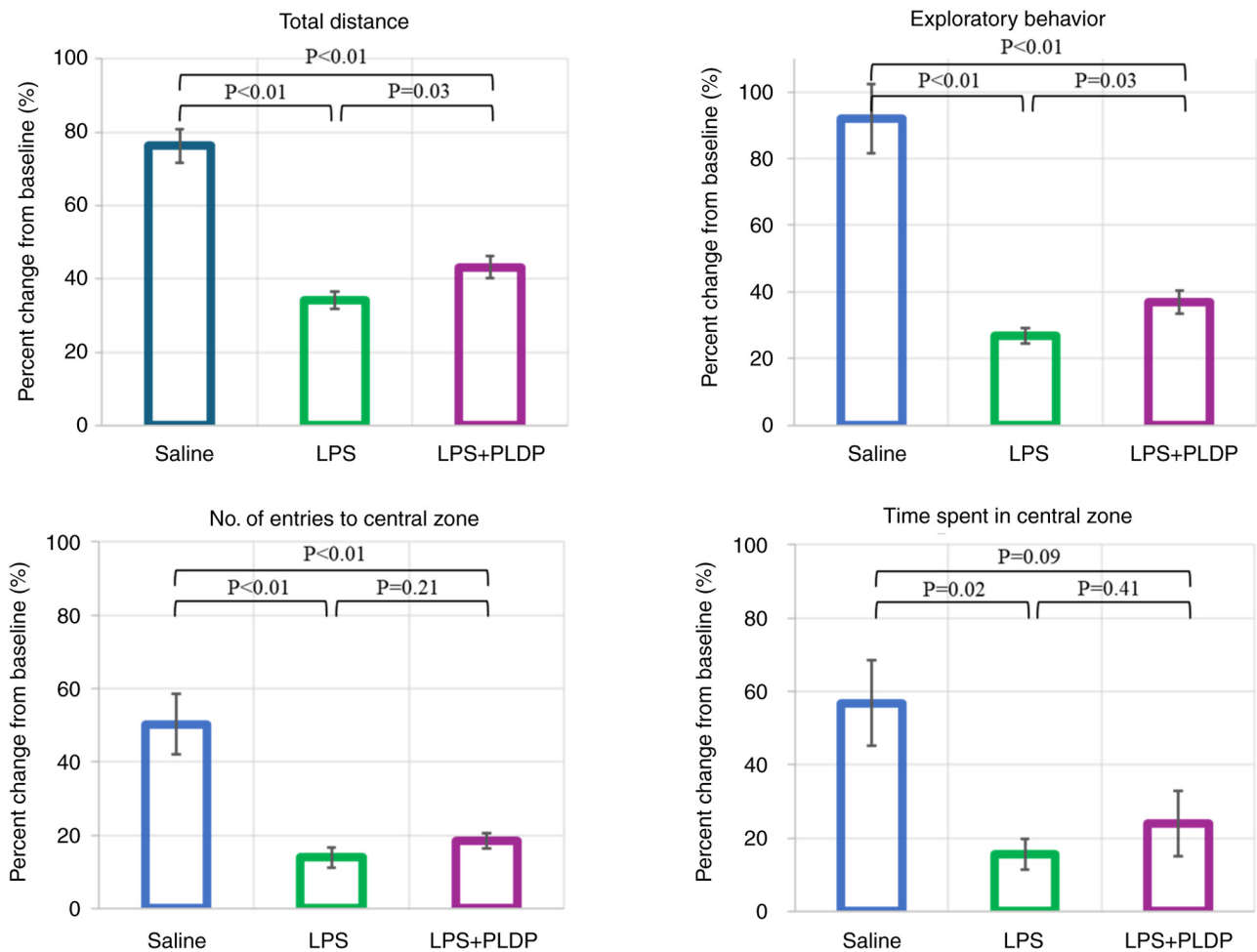


Figure 4. Percentage change from day-one baseline in the mean total distance traveled, number of exploratory behaviors, number of central entries, and time spent in the central zone on the second day of the OFT in the LPS-induced inflammation model. PLDP or saline were orally administered daily for 5 days prior to LPS administration. The LPS group received saline + LPS, and the PLDP group received PLDP + LPS. Data are expressed as the mean $\pm$ SE ($n=8$). OFT, open field test; LPS, lipopolysaccharide; PLDP, porcine liver decomposition product.

symptoms such as fever and abnormal psychological and physical symptoms referred to as depressive symptoms. Early symptoms include a loss of appetite and fatigue, with cognitive impairment manifesting during the later stages (19).

Shirato and Kizaki (20) reported that the SARS-CoV-2 spike protein S1 subunit activates macrophage signaling through toll-like receptor (TLR) 4, such as LPS, and promotes inflammatory cytokine production [IL-6 and tumor necrosis factor (TNF)- α] (20). Similarly, Khan *et al* (21) reported that the SARS-CoV-2 spike protein S1 subunit activates TLR2 and the NF- κ B signaling pathway, thereby enhancing inflammatory cytokine production (IL-1 β , IL-6 and TNF- α) (21). These findings indicate that the SARS-CoV-2 spike protein triggers inflammatory pathology through TLR4 and TLR2.

Yamato *et al* (22) induced neuroinflammation in rats by intraperitoneally injecting polyriboinosinic acid: polyribocytidylic acid (poly I:C), a TLR3 agonist that mimics viral infection, and examined the mechanism by which neuroinflammation transitions from an acute to a chronic condition. Rats exhibit transient fever and a prolonged suppression of spontaneous activity several days after poly I:C injection, and although fever is suppressed by administering a cyclooxygenase-2 inhibitor (NS-398), spontaneous activity

is not improved, suggesting that the reduction in activity is not induced by the arachidonic acid cascade that causes fever (22). Furthermore, IL-1 β and IL-1 receptor antagonist (IL-1ra) are overexpressed in the brain, including in the cerebral cortex, following poly I:C treatment. When IL-1ra is administered intracerebroventricularly to inhibit IL-1 receptors in the brain, the suppression of spontaneous activity by poly I:C is completely attenuated. Moreover, fatigue-like behavior is induced by suppressing the serotonergic system, while the amplification of IFN- α expression in the brain is also suppressed (22). These results suggest that IL-1 β is a key trigger of neuroinflammation and that IL-1ra suppresses chronic neuroinflammation (22). Meanwhile, Li *et al* (23) quantitatively evaluated brain inflammation in a rat model of virus infection using PET imaging and reported that inflammatory cytokine levels in the blood increase significantly 2 h after the intraperitoneal administration of poly I:C and that fever persists for several hours, accompanied by significant decreases in spontaneous activity, which gradually recover over several days. This indicates that fatigue persists even after the acute symptoms of fever subside. Moreover, imaging using the PET probe [18F]DPA-714, which specifically recognizes activated microglia in the brain, reveals

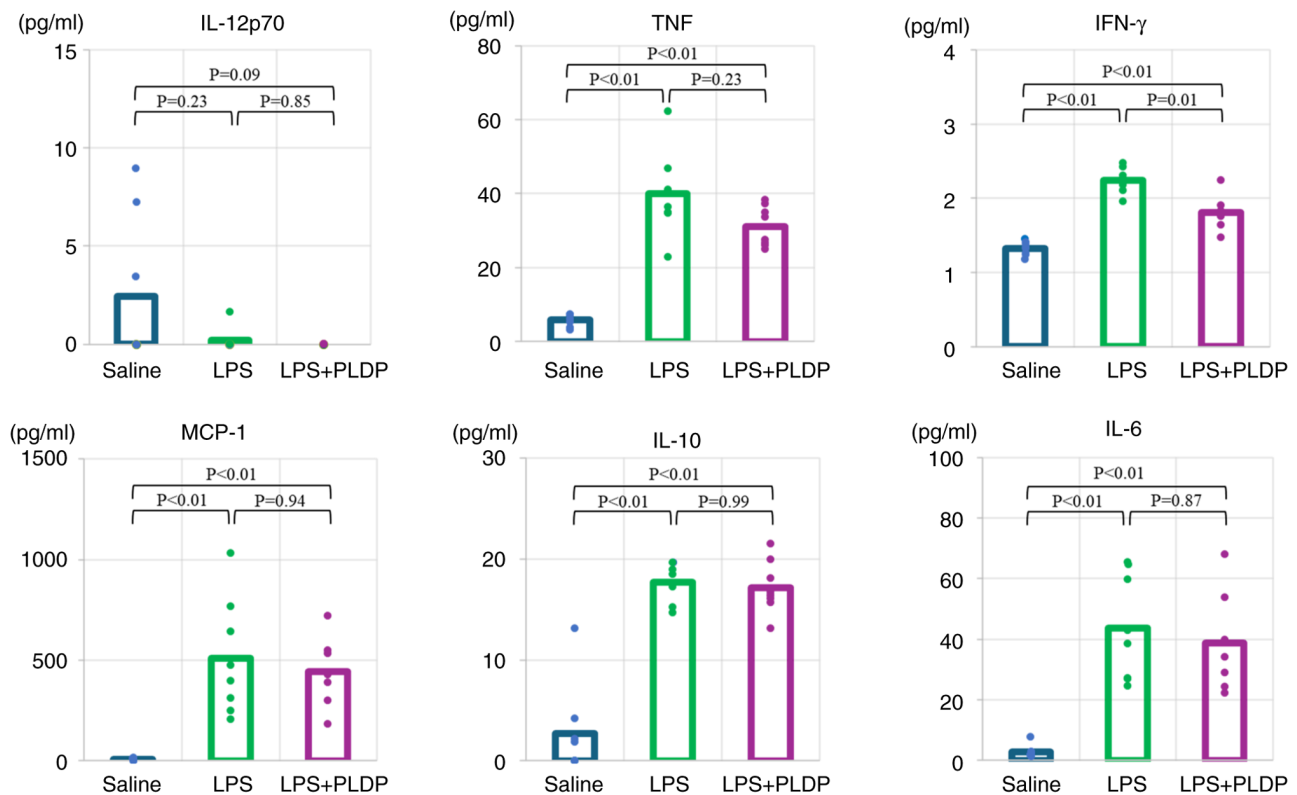


Figure 5. Percentage change from day-one baseline in plasma cytokine levels on the second day of the LPS-induced inflammation model. PLDP and saline were orally administered daily for 5 days prior to LPS administration. The LPS group received saline + LPS, and the PLDP group received PLDP + LPS. Data are expressed as the mean $\pm$ SE (n=8). LPS, lipopolysaccharide; PLDP, porcine liver decomposition product.

significant increases in brain inflammation in the nucleus tractus solitarius and parabrachial nucleus, which are vagus nerve afferents. Additionally, inflammatory cytokines released from peripheral tissues stimulate cerebrovascular endothelial cells and vagus nerve afferents, inducing inflammation in the brain, similar to an actual viral infection (23). Furthermore, correlation analysis between [18F] DPA-714 accumulation in areas where intracerebral inflammation is observed and the decrease in spontaneous activity (fatigue) reveals a positive correlation between fatigue and intracerebral inflammation in the dorsal raphe nucleus (and other areas). This suggests a potential relationship with intracerebral inflammation-induced fatigue and serotonin neurons, which are abundant in the dorsal raphe nucleus (23).

These studies suggest a mechanism that explains the relationship between intracerebral inflammation and fatigue due to viral infection. Viruses that infect peripheral tissues may cause inflammation in the dorsal raphe nucleus of the brain. This in turn can cause abnormalities in serotonin nerve function due to intracerebral inflammation. Finally, the abnormalities in serotonin nerve function may ultimately cause long-term fatigue.

Although the detailed mechanisms underlying long COVID and brain fog have not yet been elucidated, 'neuroinflammation in the brain' has been identified as a key factor underlying their pathology. The current findings demonstrated that PLDP and the LPS-induced inflammation model can be used as an experimental system to provide important insights into the pathology of long COVID and

brain fog. The present study is considered the first in that regard and that PLDP, which was demonstrated in this model to suppress abnormal behavior and inflammatory cytokine production, may be effective against long COVID and brain fog. Moreover, significant suppression of IFN- γ production was observed in the present study; however, only a tendency for suppression was shown for other inflammatory cytokines. Our future studies will examine the expression pattern of each cytokine in more detail, as well as the effects of PLDP dosage, and the timing of PLDP administration and blood collection.

OFT evaluates spontaneous activity and anxiety-like behavior in animals in a novel environment (24). In the present study, the LPS + PLDP group showed significant improvements in spontaneous motor activity and exploratory behavior compared with the LPS group. However, no significant differences were observed in other indices. Mice naturally exhibit 'thigmotaxis', a tendency to stay close to the walls the open field (where the body touches the wall), which limits their exploratory behavior toward the center. As a result, it is difficult to accurately assess the effects of PLDP. Nevertheless, the observed improvements in spontaneous motor activity and exploratory behavior may be attributed to the anti-neuroinflammatory effects of PLDP. Altering the dosage and timing of PLDP administration, as well as the duration of OFT, may potentially influence exploratory behavior toward the center.

The optimal dose and duration of PLDP administration remain undetermined. To the best of our knowledge, this

is the first *in vivo* study to investigate the effects of PLDP. Wakasugi *et al* (16) demonstrated that oral administration of oleacein, a rare component found in olives, for 10 consecutive days followed by intraperitoneal injection of LPS resulted in the suppression of depression-like behavior in mice. Similarly, Hong *et al* (17) reported comparable findings using cinnamon water extract, which was administered for 6 days. Based on these prior studies indicating that even short-term administration can exert beneficial effects on inflammation-induced behavioral abnormalities, a 5-day administration period was selected for PLDP in the present study. By contrast, clinical trials have reported memory-enhancing effects and improved prefrontal cortex function following longer-term administration of PLDP, typically over 2 to 4 weeks. These findings suggest that the optimal dosage and duration of PLDP administration may vary depending on the specific experimental context and outcome measures. Therefore, future studies should aim to determine the most effective dose and treatment duration of PLDP and PEL using both formalin- and LPS-induced inflammation models. These considerations represent important limitations of the present study and should be addressed in subsequent research.

Identifying the main targets of the gene signaling pathways modulated by PLDP and PEL would provide valuable insights into their mechanisms of action. In this context, it has been previously demonstrated that lyso-phosphatidylethanolamine (LPE) activates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 pathway. Furthermore, in cultured cortical neurons, LPE was shown to promote neurite outgrowth via multiple signaling cascades, implicating the involvement of G protein-coupled receptors in these processes (25). Additionally, it was hypothesized that PEL may inhibit TLR4-NF- κ B signaling and promote M2 polarization via STAT6 (26).

Future studies focusing on the transcriptional regulation associated with these pathways are warranted to elucidate the precise molecular mechanisms underlying the effects of PLDP and PEL.

In summary, the present findings suggest that PEL contained in PLDP is a potential functional nutrient that may alleviate long COVID and brain fog by suppressing the production of inflammatory cytokines and reducing the inflammatory response. PLDP was demonstrated to exert anti-inflammatory effects in a systemic inflammation model by shifting macrophages to the M2 phenotype. In addition, PLDP exhibited a substantial central neuroprotective effect, suggesting that it may alleviate brain fog caused by COVID-19, potentially via PEL contained in PLDP as the active component. However, these results require validation, and further research is required to explore the potential role of other PLDP components.

The present study has several limitations. First, the optimal dose and duration of PLDP administration may vary depending on the experimental conditions. The behavioral abnormalities induced by formalin- and LPS-induced inflammation performed in the present study may be experimental systems in which the effects are relatively easy to detect. Second, the timing of evaluations for outcome measures such as behavioral characteristics and cytokine production may not have been optimal, raising the possibility that only a subset of PLDP's effects was captured. Third, the long-term

effects of PLDP and its clinical relevance remain elucidated. Comprehensive evaluations, including extended observation periods and clinical studies, are essential for drawing definitive conclusions regarding its therapeutic potential. Nevertheless, the present study demonstrates that PLDP exerts measurable effects within a relatively short period in both the formalin-induced peripheral inflammation model and the LPS-induced neuroinflammation model. These findings suggest that these models are valuable tools for investigating the pharmacological actions of PLDP in greater detail, including its long-term effects and potential clinical applications. Despite limitations, our findings support the potential of PLDP as a promising strategy for neuroinflammatory conditions such as brain fog and related cognitive dysfunctions associated with COVID-19.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

IS and YM conceptualized the study. TM, SK, TeK, TaK and TTs developed methodology. ToT performed software analysis. HH, TaT and YM validated data. IS and ToT performed formal analysis. YM conducted investigation and provided resources. IS curated data. IS and YM prepared the original draft. YM and TTs wrote, reviewed and edited the manuscript and acquired funding. IS and TM visualized data. YM supervised the study and conducted project administration. IS and YM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was planned and approved in accordance with the animal experiment regulations of Nihon Pharmaceutical University (approval nos. AE2023-005 and AE2024-017; Saitama, Japan). The present study was conducted at the Kyoto Institute of Nutritional Pathology with the approval of the Animal Ethics Committee of the Kyoto Institute of Nutritional Pathology (approval date: May 7, 2024; approval no. 23027NY②; Kyoto, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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