The Combined Effects of Lysophospholipids against Lipopolysaccharide-induced Inflammation and Oxidative Stress in Microglial Cells

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Abstract: Lysophospholipids (LPLs) are small bioactive lipid molecules characterized by a single carbon chain and a polar head group. LPLs have recently shown to be involved in many physiological and pathological processes such as nervous system regulation. In our previous studies, a porcine liver decomposition product (PLDP) has been identified as a substance that improves cognitive function at old ages. This PLDP is a rich source of LPLs, including lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). This study was designed to evaluate the anti-inflammatory effect of these LPLs on lipopolysaccharide (LPS)-stimulated SIM-A9 microglial cells in terms of cytokine expression and oxidative stress and to investigate the potential mechanisms underlying these effects. SIM-A9 cells were pretreated with LPLs prior to LPS stimulation, and the anti-inflammatory potential of the LPLs in LPS-induced SIM-A9 cells was examined. Pretreatment with LPLs significantly inhibited the LPS-induced expression of IL-6 in SIM-A9 cells. Furthermore, oxidative-related protein, NADPH oxidase 2 (Nox2) levels were markedly increased in the LPS-treated cells, and pretreatment with LPC and LPE significantly reduced to basal levels. In addition, LPS-induced ROS production was eliminated in apocynin-treated cells, indicating that ROS production was dependent on Nox2. Our findings revealed that pretreatment with LPC and LPE decreased LPS-stimulated ROS production. These results indicated that LPC and LPE exerted significant protective effects against LPS-induced inflammation and oxidative stress in SIM-A9 cell.

Key words: microglia, lysophospholipid, lipopolysaccharide, Nox, oxidative stress, reactive oxygen species

1 Introduction

Neurodegenerative diseases share many common characteristics such as changes in microglial number and morphology, oxidative stress, elevated cytokine levels, and progressive neuronal loss¹. Increasing evidence suggests that microglia could be a chronic source of reactive oxygen species (ROS) and cytokines that drive progressive neuronal damage, and they are implicated in the chronic nature of neurodegenerative diseases³. Microglia activation is a hallmark of brain pathology⁴. We reported that a porcine liver decomposition product (PLDP) improves cognitive function at old age⁵, and composition of this PLDP has been well characterized⁶. The PLDP is a rich source of phospholipids, but the specific mechanism through which it affects cognitive function remains unclear. In this study, the effect of LPLs on oxidative stress and cytokine production was examined in lipopolysaccharide (LPS)-activated spontaneously immortalized SIM-A9 microglial cells. LPS is widely used as a pro-inflammatory stimulus for microglia both in vitro and in vivo⁶,⁷. Hence, suppression of microglial activation has been applied as an approach for treating such diseases. In the present study, neuroinflammation was induced by LPS in microglial cells. Furthermore, the anti-inflammatory effect of LPLs against LPS-activated microglial cells was evaluated by analyzing the expression of inflammatory mediators, including cytokines. We found that synergistic suppressive effect of combined LPE and LPC treatment on LPS-mediated interleukin-6 (IL-6) expression. Our results showed that LPS-induced IL-6 expression is responsible for the activation of nicotinamide adenine dinucleotide phosphate oxidase (Nox) in microglial cells, and this activation was significantly inhibited.

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Accepted March 30, 2021 (received for review February 17, 2021)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjos
by lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). In brain tissues after ischemic stroke, Nox is considered as a major source of ROS, which are known to cause oxidative damage to various cells and induce organ dysfunction. Our study also indicated that LPC and LPE synergistically suppressed LPS-stimulated ROS production and exerted significant inhibitory effect. These results indicated that LPC and LPE exerted significant protective effects against LPS-induced inflammation and oxidative stress in SIM-A9 cell.

2 Materials and Methods

2.1 Reagents
LPS (from E. coli O26 by phenol extraction) was purchased from FUJIFILM Wako (Tokyo, Japan). LPC18:1 and LPE18:1 were purchased from Avanti Polar Lipids (Alabaster, AL, USA). CD11b antibody (ab13357) and apocynin were purchased from Abcam (Cambridge, MA, USA). Iba1 antibody (MABN92) was purchased from Sigma-Aldrich (St. Louis, MO, USA). β-actin (sc-47778) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell lines and culture conditions
The microglial cell lines SIM-A9 were cultured in Dulbecco’s modified Eagle medium (FUJIFILM Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Nacalai, Kyoto, Japan). The cells were maintained at 37°C in a humidified atmosphere with 5% CO2, and the growth medium was refreshed every two days until the cells reached confluence.

2.3 Measurement of cell proliferation
SIM-A9 microglial cells were seeded in 96-well plates and pretreated with phospholipids at various concentrations (0, 1, 3, 10, and 30 μM), followed by LPS treatment for 24 h. Thereafter, 10 μL of Cell Counting Kit-8 (WST-8 assay) solution was added to each well. The plates were then incubated for 2 h and orange formazan dye levels were determined by measuring the absorbance at 450 nm using a microplate reader (Awareness Technology, Westport, CT, USA).

2.4 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)
Cells were seeded in 12-well plates at 1 × 103 cells/well and pretreated with phospholipids at various concentrations, followed by LPS treatment for 24 h. Total RNA was extracted from the cells using TRIzol (Invitrogen) and a NucleoSpin RNA II Kit (Takara, Otsu, Japan). Next, 0.5 μg of total RNA was converted into cDNA using a ReverTra Ace qPCR RT Kit (Toyobo) according to the manufacturer’s instructions. mRNA levels were quantified using the Quantstudio 12K Flex Real-time PCR system (ThermoFisher Scientific) or MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.). All PCR reactions were performed in 384-well PCR plates (FrameStar, 4titude) or single tubes, using GeneAce SYBR qPCR MIX α with low ROX (Nippon Gene, Toyama, Japan) or without ROX and the specified primers listed in Table 1. The reaction conditions were as follows: 95°C for 10 min (polymerase activation), followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. After amplification, the samples were slowly heated from 55°C to 95°C, and fluorescence was measured continuously to obtain a melting curve. Relative mRNA levels were quantified using the formula 2^ΔΔCq, where ΔCq is the difference between the threshold cycle of a target cDNA and an en-

Table 1 List of forward primers and reverse primers used for amplification using real-time PCR.

| Gene Name | Primer (Forward) | Primer (Reverse) |
|-----------|------------------|------------------|
| β-actin   | GGCCTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| Nox1      | AGTACGCTGACGCTTCA   | CCAATGTCGAGCCTTCA |
| Nox2      | AGTACGCTGACGCTTCA   | CCAATGTCGAGCCTTCA |
| Nox3      | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| Nox4      | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| IL-6      | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| IL-1β     | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| TNF-α     | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| IL-10     | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| Iba-1     | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |
| CD11b     | CAGTGATTCCTCCTCATCG | CCAATGTCGAGCCTTCA |

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dogenous reference cDNA.

2.5 Western blot

Cells were seeded in 6-well or 12-well plate and pre-treated with indicated compounds at various concentrations, followed by LPS treatment for 12 or 24 h. The cells were lysed with RIPA buffer (ATTO, Tokyo, Japan), and protein concentration was determined using the Bradford method (Dojindo, Japan). Proteins were separated by 4–20% SDS-PAGE and electro-transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in Block Ace (DS Pharma Biomedical, Osaka, Japan) for 1 h and incubated for 12 h at 4°C with a 1:2,000 dilution of the primary antibody in Tris-buffered saline-Tween 20 containing 5% Block Ace. Unbound primary antibodies were washed off and the membranes were incubated with 1:10,000 dilution of the horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized with Pierce ECL (Thermo Fisher Scientific) and images were obtained using a ChemiDoc Touch Imaging System (Bio-Rad).

2.6 ROS measurement using dihydroethidium (DHE)

Intracellular ROS levels were analyzed using a MUSE oxidative stress kit (Millipore) according to the manufacturer’s instructions. Cells were seeded in 6-well plates (Corning, NY, USA) at a density of 5 × 10⁴ cells/mL and pretreated with indicated compounds at various concentrations, followed by LPS treatment for 2 h. The cells were incubated with DHE (Millipore) for 30 min in the dark. The assay measures the relative percentage of cells that are ROS-negative and ROS-positive by evaluating the fluorescence of DHE using flow cytometry (MUSE™ Cell analyzer) or fluorescent plate reader (Bio Tek’s).

2.7 Small interfering RNA

The expression of Nox2 was inhibited by transfecting SIM-A9 cells with an Nox2-targeting small interfering RNA (siRNA; Nippon Gene), while a scrambled siRNA was used as a control. The siRNAs used in this study were synthesized by Nippon Gene. The sequences were as follows: GAA ACUACCUAAGAUGCUGGAU for Nox2. Cells were cultured in 6-well plates (Iwaki, Tokyo, Japan) at a density of 5 × 10⁴ cells/well in DMEM with 10% FBS. Cells were transfected with 100 pmol/mL of mRNA-specific siRNAs using Lipofectamine RNAimax (Invitrogen). The reduction in the Nox2 protein levels was confirmed using western blot analysis.

2.8 Statistical analysis

The data were analyzed using an unpaired Student’s t-test or one-way analysis of variance, followed by Newman–Keuls post hoc testing with GraphPad Prism Ver. 5.01 (GraphPad Software Inc.). The results are expressed as the mean ± standard error of the mean (SEM). P<0.05 was considered to indicate statistical significance.

3 Results

3.1 Microglial cell viability, morphology, and marker expression after LPS exposure

To determine the effects of LPS on neuroinflammation, we examined whether LPS is toxic to SIM-A9 microglial cells. Cells were treated with vehicle or LPS (0, 1, 3, 10, 30, or 100 ng/mL) for 24 h, and WST-8 assays were conducted. LPS did not exhibit toxicity at all doses (Fig. 1A), indicating that while LPS induced changes in cell morphology (Fig. 1B), it did not significantly affect cell viability compared to that of control cells at concentrations up to 100 ng/mL. To assess the level of the microglial activation, we examined the mRNA and protein expression of the microglial markers Iba-1 and CD11b in SIM-A9 cells. qRT-PCR and western blot demonstrated the dose-dependent effect of LPS on the mRNA and protein expression, respectively, of Iba-1 and CD11b in SIM-A9 cells (Figs. 1C and 1D). These results suggest that the expression of Iba-1 and CD11b was increased in SIM-A9 cells when microglia were in an activated state upon LPS exposure.

3.2 Effect of LPC or LPE on microglial cell viability after LPS exposure

Next, to determine the effects of LPS on neuroinflammation, we examined whether LPS is toxic to SIM-A9 microglial cells. Cells were treated with vehicle or LPS (3, 10, 30, 100, 200, and 400 ng/mL) for 24 h, WST-8 assays were conducted for determination of the number of viable cells in cell proliferation. We determined effect of LPC 18:1 and LPE18:1 on microglial cell viability under LPS exposure. To investigate the effect of LPC 18:1 and LPE18:1 on LPS-mediated stimulation, we examined whether LPC or LPE affected SIM-A9 cell growth. Cells were pretreated with vehicle (0.1% BSA in PBS), LPC or LPE for 3 h, followed by incubation with 30 ng/mL of LPS for 24 h. As shown in Figs. 2A-2C, LPC and LPE, or its combination, did not exhibit cell growth at all doses (Figs. 2A-2C). Next, we investigated the iba-1 expression of LPC and LPE in LPS-stimulated SIM-A9 cells. As shown in Fig. 2D, real-time PCR analysis demonstrated that combined with LPC and LPE reduced the expression of iba-1 mRNA.

3.3 Synergistic suppressive effect of combined LPE and LPC treatment on LPS-mediated IL-6 expression

Next, to elucidate the mechanisms responsible for the inhibitory effect of LPE and LPC on cytokine expression, we examined the mRNA expression of M1-related pro-inflammatory cytokines and M2-related anti-inflammatory cytokine by real-time PCR analysis. SIM-A9 cells were pre-

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treated with LPC and LPE for 3 h prior to incubation with LPS for 12 h. As shown in Fig. 3A, the LPS-induced mRNA expression of IL-6 was downregulated by LPC, LPE, or its combination with 30 μM of LPC and LPE. However, another pro- and anti-inflammatory cytokine including TNF-α and IL-1β was not significantly inhibited by LPC, LPE, or its combination under LPS stimulation (Figs. 3B and 3C). These results suggesting that LPC and/or LPE negatively regulated IL-6 mRNA at the transcriptional level in LPS-stimulated SIM-A9 cells. Furthermore, the anti-inflammatory cytokine, IL-10, was not significantly induced by single treatment with LPC or LPE, however, in combined treatment with LPC and LPE, markedly increased IL-10 mRNA level (Fig. 3D).

3.4 LPS stimulation increased mRNA expression of Nox2 in microglial cells

LPS reportedly induced mitochondrial dysfunction and the accumulation of oxidative damage in DNA10. In addition, Nox activation plays an important role in LPS-induced innate immunity in macrophages by triggering superoxide anion production11. We evaluated the mRNA and protein expression of Nox1, Nox2, Nox3, and Nox4 by qRT-PCR, in resting SIM-A9 cells. There was a significant difference in the expression of Nox2 in SIM-A9 cells, whereas the expression of Nox1, 3, and 4 did not differ significantly (Fig. 4A). However, whether LPS affects Nox2 expression in microglial cells remains unclear. As shown in Fig. 4B, SIM-A9 cells exposed to LPS (0, 10, 30, 60, 100 and 200 ng/mL) for 24 h showed an increase in the mRNA expression of Nox2,
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Fig. 2 The effect of LPC and LPE on cell viability and expression of microglial markers after microglial cells were exposed to LPS. (A)-(C) Viability of SIM-A9 cells after pretreatment with LPLs (LPC or LPE, individually at 0, 1, 3, 10, and 30 μM) or a combination of LPC and LPE followed by 24 h of incubation with 30 ng/mL LPS. Colorimetric cell viability assays using WST-8 with microplates were carried out. The data are expressed as the mean ± SEM, n=3. (D) LPS induced SIM-A9 cell activation and upregulation of Iba-1 levels. LPC and LPE inhibited LPS-induced Iba-1 expression in SIM-A9 cells.

Fig. 3 LPC and LPE inhibited LPS-induced cytokine expression. (A-D) The mRNA level of cytokines (IL-6, TNF-α, IL-1β, and IL-10) in SIM-A9 cells. Cells were pretreated with LPC and LPE (10 and 30 μM) for 3 h, followed by 12 h of incubation with 30 ng/mL LPS. Real-time PCR was performed by using specific primers for L-6, TNF-α, IL-1β, and IL-10. The data are expressed as the mean ± SEM, n=3, **p<0.01.
suggesting that LPS could be responsible for the mRNA expression of Nox2 in the cells. A previous report suggested that the phospholipid phosphatidic acid stimulated Nox activity in neutrophils. To examine whether LPC and LPE affect Nox2 expression in microglia, we treated LPS-exposed SIM-A9 cells with LPC or LPE at 10 or 30 μM. As shown in Fig. 4C, Nox2 levels were markedly increased in the LPS-treated cells, and pretreatment with LPC and LPE significantly reduced Nox2 mRNA levels.

3.5 Effect of LPS stimulation on ROS generation in microglial cells

It has been reported that LPS is not directly toxic to neurons but activates microglia to produce ROS through
inducible nitric oxide synthase\(^{10}\) and Nox\(^{14}\), and the role of Nox2 in LPS-induced neurotoxicity has been confirmed in vivo\(^{20}\). We next determined the effect of LPS stimulation on ROS generation in microglial cells. As shown in Fig. 4D, the increase in DHE fluorescence indicates that LPS induced the production of significant amounts of intracellular ROS. To examine the inhibitory effects of Nox, we used 10 μM apocynin, a selective inhibitor of Nox with an IC\(_{50}\) of 10 μM in activated human neutrophils\(^{10}\), as a positive control. The increase in ROS was eliminated in apocynin-treated cells, indicating that ROS production was dependent on Nox. As shown in Fig. 4E, treatment with the control siRNA significantly increased apocynin-mediated intracellular ROS production. Nox2 siRNA extensively prevented the induction of ROS generation, thus validating our assumption that Nox2-mediated ROS production may be the main cause of inflammation induction in SIM-A9 cells. Collectively, the results suggest that LPS induced Nox-dependent ROS generation in microglial cells.

### 4 Discussion

Inflammatory response is crucial in controlling and counteracting the harmful effects triggered by a variety of insults to the central nervous system. However, severe or chronic neuroinflammation can damage the central nervous system because of excessive production of cytokines and other inflammatory mediators, such as ROS, by microglia\(^{17}\). Here, we identified novel cooperative actions of LPLs that inhibit IL-6 expression and intracellular ROS accumulation in microglia after LPS-induced neuroinflammation. LPS, a prominent component of the cell wall of gram-negative bacteria, is a strong stimulator of microglial activation\(^{18}\). Once activated, microglia rapidly undergo morphological changes characterized by cell body enlargement\(^{29}\). To validate our previous findings, we first assessed whether and how LPS administration affects the size and viability of microglial cells and the expression of microglial markers. In this study, we focused on newly identified LPLs that are produced by activated platelets, damaged cells, and cells that have been stimulated with growth factors\(^{20}\). Over the past several decades, various molecular pathways that regulate microglial activation and the effect of activated microglia on neurons have been identified. The current results showed that LPS-mediated inflammatory response, including the increase in microglial cytokine production, was suppressed by LPC or LPE exposure. In addition, we observed the synergistic effects of LPC and LPE in microglial cells under LPS treatment. The combined treatment of LPC and LPE dramatically inhibited the LPS-induced mRNA expression of IL-6. This finding is important given that microglia-mediated neuroinflammation is regarded as a pathological mechanism in many neurodegenerative diseases, such as dementia and Alzheimer’s disease, and a key event in accelerating cognitive or functional decline\(^{21}\). In addition, metabolomics approaches are promising tools for studying synergy, and have just begun to be applied to identifying constituents that participate in combination effects\(^{22}\). By understanding the synergistic activity of LPLs within PLDP extracts, we will be able to optimize the safety and efficacy of treatments schemes for neurological disorders and diseases. Our results also revealed that microglial cells showed increased expression of the anti-inflammatory cytokine IL-10, and its regulation involves the pro-inflammatory cytokine IL-6. The pro-inflammatory cytokines IL-6 and TNF-α have been demonstrated to induce microglial IL-10 production in a dose-dependent manner\(^{23}\). IL-10, initially identified as a T-cell product that inhibits the synthesis of pro-inflammatory cytokines, is produced upon the activation of different cell types, including microglia. Furthermore, we found that the LPS/IL-6/Nox2 axis plays a key role in biological processes and regulates continuous ROS production in microglial cells. Nox2 is the major isoform of Nox found in the microglial cell membrane\(^{24}\). Our results suggest that the increase in Nox2-derived oxidative stress in microglia could be involved in neuropathological pathways. Although the exact mechanism of this phenomenon remains unclear, we have conducted investigations on functional phospholipids. A recent report also indicated that LPC in PLDP inhibited microglia activation\(^{23}\). We aimed to identify a functional phospholipid contained within PLDP that has been confirmed to have clinical effects and to develop a new drug for treating neurodegenerative disease.

### Conflict of Interest

The authors declare no competing financial interests.

### Acknowledgments

This work was supported by the Japan Society for the Promotion of Science KAKENHI[grant number 20K09476 and 16K15660] to Hisao Haniu and Tamotsu Tsukahara, and by The Ito Foundation[grant number ken-1] to Tamotsu Tsukahara. This work was performed using research equipment shared by the MEXT Project for promoting public utilization of advanced research infrastructure (Program for supporting introduction of the new sharing system, grant number JPMXS04225000320).

### Author Contributions

Tamotsu Tsukahara, Hiroto Hara, Hisao Haniu, and Yo-
shikazu Matsuda conceived and designed the project. Hiroto Hara and Tamotsu Tsukahara acquired the data. Hiroto Hara and Tamotsu Tsukahara analyzed and interpreted the data. Tamotsu Tsukahara wrote the paper.

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