Structurally different lysophosphatidylethanolamine species stimulate neurite outgrowth in cultured cortical neurons via distinct signaling pathway

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Research

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Abstract

Neurite outgrowth is important in neuronal circuit formation and functions, and for regeneration of neuronal networks following trauma and disease in the brain. Thus, identification and characterization of the molecules that regulate neurite outgrowth are essential for understanding how brain circuits form and function and for the development of treatment of neurological disorders. In this study, we found that lysophosphatidylethanolamine (LPE), one of the lysophospholipids, influences neurite outgrowth in cultured cortical neurons. Extracellular application of either of the structurally different LPE species, palmitoyl LPE (16:0 LPE) and stearoyl LPE (18:0 LPE) dramatically increased the areas of axon and dendrite without affecting the neuronal viability. Subsequent analysis revealed that both LPEs increased the length of neurite in a dose-dependent manner. Interestingly, inhibition of phospholipase C, one of the effectors for G-protein-coupled receptor-mediated signaling pathways, inhibited 18:0 LPE-stimulated neurite outgrowth but not 16:0 LPE-stimulated neurite outgrowth. The effects of protein kinase C (PKC) inhibitors on neurite outgrowth were also different. Inhibitor against PKCα, β, δ, ε, η, and θ inhibited both 16:0 LPE- and 18:0 LPE-induced neurite outgrowth. In contrast, an inhibitor against PKCα, β, γ, δ, and ζ inhibited the 18:0 LPE effect but not the 16:0 LPE effect. We also found that both 16:0 LPE and 18:0 LPE activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)1/2. There was no substantial difference in the amount of phosphorylated MAPK/ERK1/2 between 16:0 LPE and 18:0 LPE-treated cultures. MAPK inhibitor completely inhibited 18:0 LPE-induced neurite outgrowth and partially inhibited 16:0 LPE-induced neurite outgrowth. Thus, the effect of the MAPK inhibitor differed between the 16:0 LPE- and 18:0 LPE-treated cultures. Collectively, the results suggest that the structurally different LPE species, 16:0 LPE and 18:0 LPE stimulate neurite outgrowth through the distinct signaling cascades in cultured cortical neurons.

Introduction

Neurite outgrowth is important in neuronal wiring during development and synaptic plasticity under physiological conditions, and in the regeneration of neuronal wiring following trauma and disease in the brain [1–4]. Thus, identifying the molecules that regulate neurite outgrowth and elucidating their mechanism are essential for understanding of mechanisms of neuronal wiring and brain functions, and for development of therapeutics to treat trauma or neurological disorders. To date, several extracellular ligands that regulate the neurite outgrowth of central nervous system (CNS) neurons have been identified. These ligands are varied and include neurotrophins, Wnt proteins, neurotransmitters, neuropeptides, and hormones [5–11].

Phospholipids are amphiphilic molecules with a hydrophilic head esterified by a hydrophobic fatty acid chain. They play crucial roles in various biological processes, ranging from structural integrity of cellular and organelle membranes, to intracellular signal transduction [12]. The brain is one of the most lipid-rich organs, and phospholipids especially play important roles not only in membrane organization but also in
brain function [3, 13, 14]. Among the physiologically active phospholipids, the role of lysophospholipids in biological functions has attracted recent attention. Lysophospholipids consisted of a hydrophilic head group and one hydrophobic fatty acid chain, and many lysophospholipid molecular species exist. Lysophosphatidylethanolamine (LPE) is one of the lysophospholipids derived from phosphatidylethanolamine (PE) via a phospholipase A-type reaction and is known to be a minor constituent of cell membranes [15, 16]. As for other lysophospholipids, LPE presents many structurally different species that differ in fatty acid length and degree of unsaturation [15]. Extracellularly, LPE has been detected in human serum at levels of several hundred nanograms per milliliter [17], but its physiological role remains largely unknown. A few studies suggest the cell-type specific roles of LPE in cultures [18–21]. Interestingly, several studies show that cognitive impairment or traumatic brain damage in rodent model is accompanied by changes in LPE revelations in the brain [22–24]. These suggest the physiological or pathological significance of LPE in the brain. However, to date, the role of LPE in CNS neurons remains unclear. In this study, we demonstrate that LPEs stimulate neurite outgrowth in the cultured cortical neurons. Moreover, using several enzyme inhibitors, the structurally different LPE species, palmitoyl LPE (16:0 LPE) and stearoyl LPE (18:0 LPE) were found to stimulate neurite outgrowth through distinct signaling cascades.

Results

Egg yolk LPE increase axon and dendritic area in cultured cortical neurons

To examine the effects of phospholipids on neuronal morphology, a commercially available phospholipid kit containing 10 different phospholipids (see the Methods for detail) was applied to cultured cortical neurons prepared from mouse embryos. The phospholipids bovine liver lysophosphatidylcholine (LPC), egg yolk LPE, soybean phosphatidylinositol (PI), bovine heart sphingomyelin (SM), porcine brain phosphatidylserine (PS), bovine heart phosphatidylcholine (PC), egg yolk phosphatidylethanolamine (PE), bovine heart cardiolipin (CL), phosphatidic acid (PA) prepared from egg yolk PC, and porcine brain cerebrosides (CB) were applied to a cultured medium at days in vitro (DIV) 3. At DIV14, microtubule-associated protein 2 (MAP2)-positive dendrites and microtubule-associated protein tau (tau)-positive axons had spread all over the dish in the control cultures (Fig. 1a). When the cultured cortical neurons were incubated with 1 µM egg yolk LPE for 11 days, the tau and MAP2 signals area were significantly increased compared to those in the control cultures (Fig. 1a-c). These results suggest that egg yolk LPE strongly stimulates both axonal and dendritic outgrowth in cultured cortical neurons. In cultures treated with soybean PI and bovine heart PC, MAP2 and tau signals were slightly increased compared to those in the control cultures. On the other hand, there was no substantial difference in the number of neuronal nuclear antigen (NeuN)-positive neurons between the control and the cultures treated with phospholipids, except for treatment with bovine heart CL (Fig. 1a and d). In the bovine heart CL-treated cultures, the number of NeuN-positive neurons, the tau and MAP2 signal areas were significantly decreased.

16:0 LPE and 18:0 LPE increase axon and dendritic area in cultured cortical neurons
Among phospholipids tested, egg yolk LPE was the most effective on the morphological change. To our knowledge, no previous study examined the role of LPE in the CNS neurons. Therefore, LPE effects were examined in this study. The LPE used was derived from egg yolk and contained two structurally different LPE species, 16:0 LPE and 18:0 LPE. To examined whether 16:0 LPE or 18:0 LPE effects the neuronal morphology, cultured cortical neurons were incubation with different concentrations of LPE, 16:0 LPE, and 18:0 LPE for 11 days. Incubation with 0.1 or 1 µM 16:0 LPE significantly increased the tau and MAP2 signal areas compared to those in the control cultures (Fig. 2a-c). At a higher concentration of 10 µM, 16:0 LPE did not significantly increased these signal areas. On the other hand, 18:0 LPE significantly increased the tau and MAP2 signal areas at all concentrations (Fig. 2a-c). There was no substantial difference in the number of NeuN-positive neurons when comparing the control and the LPEs-treated cultures (Fig. 2a and d). These results suggest that the structurally distinct LPE species 16:0 LPE and 18:0 LPE both increase axonal and dendritic area in cultured cortical neurons.

**16:0 LPE and 18:0 LPE stimulate neurite outgrowth in cultured cortical neurons.**

The effects of 16:0 LPE and 18:0 LPE on neurite outgrowth were examined by directly measuring the length of neurites. Either 16:0 LPE or 18:0 LPE was applied at 1 µM to the culture medium at DIV0, and the longest neurites length was measured at DIV1, 2, and 3, respectively. From DIV0 to DIV3, the lengths of neurites were gradually increased in all conditions. In cultures treated with 16:0 LPE and 18:0 LPE, the neurites were significantly longer than those of control cultures (Fig. 3a and b). The number of neurites emerging from the soma was also increased in these cultures at DIV1 and 2 (Fig. 3a and c). On the other hand, the numbers of branches for the longest neurite in 16:0 LPE- and 18:0 LPE-treated cultures were comparable to those in the control culture (Fig. 3a and d). In terms of morphologies examined, there was no substantial difference between the 16:0 LPE- and 18:0 LPE-treated cultures. The effects of different concentrations of 16:0 LPE and 18:0 LPE were also examined in terms of neurite outgrowth. The cultured cortical neurons were incubated with 0.5 µM to 10 µM 16:0 LPE or 18:0 LPE, and the length of the longest neurites was measured at DIV3. Both 16:0 LPE and 18:0 LPE dose-dependently increased the length of neurites (Fig. 4a and b). At a concentration of 10 µM, both LPEs increased the neurite length to approximately twice as long as that in the control cultures. There was no significant difference between 16:0 LPE and 18:0 LPE in the lengths at any concentration. These results suggest that 16:0 LPE and 18:0 LPE stimulate neurite outgrowth in cultured cortical neurons and have similar effects on neuronal morphology.

**Differential effects of PLC and PKC inhibitors on 16:0 LPE and 18:0 LPE-stimulated neurite outgrowth**

Several studies suggest that the one of actions of LPE is mediated by G-protein-coupled receptor (GPCR)-activated G protein-coupled phospholipase C (PLC) [19, 20, 25, 26]. Therefore, we examined whether the GPCR-PLC cascade mediates the actions of 16:0 LPE and 18:0 LPE. Cultured cortical neurons were incubated with PLC inhibitor U73122 at 0.5 µM. Application of U73122 decreased the length of 18:0 LPE-stimulated neurite to almost the same length as in the inhibitor-treated control cultures; this suggests that 18:0 LPE action is mediated by GPCR coupled to PLC. In contrast, U73122 had little effect on 16:0 LPE-
treated cultures (Fig. 5a and d). It is known that activation of PLC leads to production of inositol 1,4,5-trisphosphate and diacylglycerol, the activator for PKC. To examine whether PKC is involved in LPEs-stimulated neurite outgrowth, the PKC inhibitor Go6983, which inhibits PKCα, β, γ, δ, and ζ [27], was applied to the cultures. Application of 1 µM Go6983 inhibited 18:0 LPE-stimulated neurite outgrowth. In contrast, Go6983 had little effect on 16:0 LPE-treated cultures (Fig. 5b and e). It is known that PKC family proteins consist of PKCα, β, γ, δ, ε, η, θ, ζ, Mζ, and ι/λ [28]. Therefore, a different PKC inhibitor, Sotrastaurin, which inhibits PKCα, β, δ, ε, η, and θ [29], was selected. When Sotrastaurin was applied to the cultures, both 16:0 LPE- and 18:0 LPE-stimulated neurite length changes were completely inhibited (Fig. 5c and f). These results suggest that 16:0 LPE and 18:0 LPE activate distinct receptors and signaling cascades involved in the neurite outgrowth.

16:0 LPE and 18:0 LPE activate MAPK in cultured cortical neurons

It has been reported that LPE activates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 in manner that depends on cell type [18, 19, 30, 31]. However, the response of LPE in neurons remains unknown. It is known that MAPK signal cascade involved in neurite outgrowth [32]. Thus, one possibility is that 16:0 LPE and 18:0 LPE activate MAPK/ERK1/2 in the cultured cortical neurons. To examine whether this takes place, the cultured cortical neurons were incubated with 16:0 LPE or 18:0 LPE for 10 min and subjected to western blot analysis using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Anti-ERK1/2 antibody then detected the bands corresponding to the size of ERK1/2 in all conditions (Fig. 6a). In the absence of LPE, anti-phospho-ERK1/2 antibody detected bands corresponding to the size of phosphorylated ERK1/2, and incubation of 16:0 LPE and 18:0 LPE significantly increased those signals in a dose dependent manner (Fig. 6a and b). These results suggest that both 16:0 LPE and 18:0 LPE activate MAPK/ERK1/2 in cultured cortical neurons.

Differential effects of MAPK inhibitor on 16:0 LPE- and 18:0 LPE-stimulated neurite outgrowth

The finding of activation of MAPK/ERK1/2 in 16:0 LPE- and 18:0 LPE-treated cortical cultures rises the possibility that these LPEs stimulate neurite outgrowth through the activation of MAPK/ERK1/2. To examine this, the cultured cortical neurons were treated with MAPK inhibitor U0126 at 5 µM in the presence or absence of 16:0 LPE or 18:0 LPE for 3 days. This concentration of U0126 completely inhibited activation of MAPK/ERK1/2 (Fig. 7a and b). In the control cultures at DIV3, application of U0126 significantly decreased the length of neurites. In cultures treated with 18:0 LPE, U0126 significantly decreased the length of neurite to a degree comparable to that in the U0126-treated control cultures (Fig. 7c and d). In contrast, U0126 decreased 16:0 LPE-stimulated neurite length, although these were longer than in the control and 18:0 LPE-treated cultures. These results suggest that 16:0 LPE and 18:0 LPE stimulate neurite outgrowth through the activation of MAPK, and that 16:0 LPE also stimulates another signal cascade that stimulates neurite outgrowth.

Discussion
Recently, the role of lysophospholipids in biological functions has attracted attention. However, in the CNS neurons, the cellular response to extracellular lysophospholipids remains largely unclear, although some phospholipids such as lysophosphatidic acid (LPA), sphingosine-1-phosphate, and 2-arachidonoylglycerol have been reported to act extracellularly as an agonist on their membrane receptor and to regulate neuronal functions [34–37]. The present study demonstrates that extracellular application of 16:0 LPE and 18:0 LPE stimulates neurite outgrowth in cultured cortical neurons through a distinct signal cascade.

The effects of 16:0 LPE and 18:0 LPE in stimulating neurite outgrowth in cultured cortical neurons are clearly shown (Figs. 3 and 4). LPE is a minor constituent of cell membranes [15]. Extracellularly, LPE was detected in human serum in the range of several hundred nanograms per milliliter [17], but its physiological role remains largely unknown. A few studies have indicated the cell-type specific roles of LPE in culture cells, as follows: The LPE extracted from Grifola frondosa, a kind of mushroom, stimulates neurite-like outgrowth of rat pheochromocytoma PC12 cells [18]. The 18:1 LPE stimulates chemotactic migration and cellular invasion in SK-OV3 human ovarian cancer cells [19] and cell proliferation of MDA-MB-231 breast cancer cells [20]. Egg yolk LPE stimulates differentiation and maturation of mouse astrocyte in culture [21]. Thus, LPE evokes various response, depending on the cell type. At this time, whether LPE promotes neurite outgrowth in other types of CNS neurons remains unclear.

In cultured cortical neurons, 18:0 LPE-stimulated neurite outgrowth was inhibited by the application of PLC inhibitor U73122 (Fig. 5a and d), suggesting that the action of 18:0 LPE is mediated by GPCR-PLC cascade. In contrast, U73122 exerted little effect on 16:0 LPE-treated cultures. Therefore, a different type of receptor is likely to exist for 16:0 LPE. In addition to neurite outgrowth, 16:0 LPE and 18:0 LPE may have different physiological functions for cortical neurons. Further investigation is needed to clarify this. To date, receptors for LPE remains unidentified, but several studies have suggested the existence of membrane-bound receptors for different LPE species [19, 20, 25–27]. In MDA-MB-231 cells, SH-SY5Y neuroblastoma cells, and PC12 cells, 18:1 LPE stimulates intracellular Ca\(^{2+}\) concentration, and this effect is inhibited by pertussis toxin (PTX), an inhibitor of Gi/Go proteins, and antagonist of LPA receptor LPA\(_1\) [20, 25–27]. On the other hand, in SK-OV3 cells, the effect of 18:1 LPE is inhibited by PTX, but not by an antagonist of LPA receptor LPA\(_1\) or of LPA\(_1\) and LPA\(_3\) [19; 27]. In addition, in some cells, LPE stimulates intracellular Ca\(^{2+}\) concentration, but their effect is completely dependent on the fatty acid lengths of the LPE [26, 27]. For example, in PC12 cells, intracellular Ca\(^{2+}\) concentration is stimulated by 18:0 LPE and 14:0 LPE, but not 16:0 LPE [26]. LPA\(_1\) activation has been reported to induce neurite retraction in B103 neuroblastoma cells [38]. Identification of receptors for 16:0 LPE and 18:0 LPE will be needed to completely elucidate how LPE induces a signal cascade in the cortical neuron.

Many ligands including neurotrophins, Wnt proteins, neurotransmitters, hormones, and neuropeptides have been identified as stimulating neurite outgrowth of neurons via binding to their receptors [5–11], and diverse protein kinase signal cascades including MAPK and PKC are involved in this process [32]. In the cultured cortical neurons, extracellular application of both 16:0 LPE and 18:0 LPE activated
MAPK/ERK1/2 (Fig. 4). It has reported that 14:0 LPE, 16:0 LPE, and 18:1 LPE activate MAPK/ERK1/2 in PC12 cells [18] and 18:1 LPE activates ERK1/2 in human MDA-MB-231 breast cancer cell line [20]. LPE, although not reported in terms of specific type, did not activate MAPK in murine mesangial cells [30]. However, for PC12 cells, contrary to the observations noted by Nishina et al [18], there is a report that 16:0 LPE does not activate MAPK/ERK1/2 in these cells [31]. These previous studies suggest the existence of a cell-type dependent MAPK activation mechanism for LPE. Interestingly, in cultured cortical neurons, inhibition of MAPK inhibited 18:0 LPE-stimulated neurite outgrowth and partially inhibited 16:0 LPE-stimulated neurite outgrowth (Fig. 7). In addition, Sotrastaurin, which inhibits PKCa, β, δ, ε, η, and θ [29], inhibited both 16:0 LPE- and 18:0 LPE-induced neurite outgrowth. In contrast, Go6983, which inhibits PKCa, β, γ, δ, and ζ [28], inhibited the effect of 18:0 but not the effect of 16:0 LPE (Fig. 5b, c, e and f). Combining these results with the PLC inhibitor results (Fig. 5a and d), suggests that 16:0 LPE and 18:0 LPE stimulate neurite outgrowth in the cortical neurons through different receptors and signaling pathways.

Thus far, the roles of LPE in the brain has not been fully elucidated, but several reports suggest the pathophysiological significance of LPE. Previous reports show that cognitive impairment or traumatic brain damage is associated with an imbalance in the composition of phospholipids in the brain, such that some studies have reported changes of LPE level in the brain. Aged triple transgenic Alzheimer’s model mice harboring PS1M146V, APPSwe, and tauP301L transgenes with cognitive impairment show increased LPE in the hippocampus [39, 40]. Postischemic cognitive impairment rat model show increased LPE species, 18:1, 20:3, and 22:6 LPE, in the hippocampus [24]. Traumatic brain injury in rat transiently increases 22:6 LPE in the injury area [23]. A change in LPE was reported in the serum of a major depressive disorder patient [40]. These observations imply the importance of LPE in the brain in normal development, as well as in pathological setting. Comprehensive analysis of LPE species in the brain and investigation of their functions are necessary to understand their roles in pathophysiological conditions.

**Methods**

**Cell cultures**

Primary cortical neuron cultures were prepared from ICR mice at embryonic day 18 as previously described [41]. Briefly, the cerebral cortices were treated with phosphate-buffered saline (PBS) containing 1% trypsin (Sigma-Aldrich) and 0.1% DNase I (Sigma-Aldrich) for 5 min and dissociated by passing through a fire-polished Pasteur pipette in PBS containing 0.05% DNasel, 0.03% trypsin inhibitor (Sigma-Aldrich), and 2 mM MgCl₂. The cells were placed on 12-diameter coverslips coated with 30 µg/ml poly-L-lysine (weight 70–150 kDa; Sigma-Aldrich) and 10 µg/ml mouse laminin (Thermo Fisher Scientific) at a density of 1.0 × 10⁵ cells /well on 24-well culture plates. The cells were maintained in Neurobasal-A (Thermo Fisher Scientific) supplemented with 2% B-27 supplement (Thermo Fisher Scientific), 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.2 mM GlutaMax-I (Thermo Fisher Scientific) for 24 and then the medium was changed to the same medium without fetal bovine serum. All animal
procedures were approved by the Animal Care and the Use Committee of Shinshu University (Approval No. 290072).

**Morphological Analysis**

Phospholipid kit including bovine liver LPC containing primary C18:0, egg yolk LPE containing primary C16:0 and C18:0, soybean PI containing primary C16:0 and C18:2, bovine heart SM containing primary C16:0, C18:0, and C18:1, porcine brain PS containing primary C18:0 and C18:1, bovine liver PC containing primary C18:0 and C18:1, egg yolk PE containing primary C16:0, C18:0, C18:1, and C18:2, bovine heart CL containing primary C18:2, PA from egg yolk PC containing primary C16:0 and C18:1, and porcine brain CB containing primary C22:0 and C24:0 were purchased from Olbracht Serdary Research Laboratories (Toronto, Canada). At DIV3, 1 µM LPC, LPE, PI, PS, PC, PE, CL, PA, and CB was applied to the culture medium, respectively, and fixed. The cultures were fixed with 4% paraformaldehyde/4% sucrose at DIV14. The fixed cells were incubated with PBS buffer containing 0.25% Triton X-100 for 5 min and immunostained with rabbit anti-MAP2 (1:1000; Sigma-Aldrich), goat anti-tau (1:1000; Santa Cruz Biotechnology), and mouse anti-NeuN (1:500; Merk Millipore) antibodies, followed by incubation with donkey Alexa Fluor 488-conjugated anti-goat IgG (1:500; Thermo Fisher Scientific), donkey Alexa Fluor 555-conjugated anti-rabbit IgG (1:500; Thermo Fisher Scientific), and donkey Alexa Fluor 647-conjugated anti-mouse IgG antibodies (1:500; Thermo Fisher Scientific). 16:0 LPE (Avanti) and 18:0 LPE (Avanti) were used to analyze the effect of the difference in the structure of LPEs. For analysis of the time course of neurite outgrowth, 1 µM 16:0 LPE or 18:0 LPE was applied to the culture medium at DIV0. The cultures were fixed with 4% paraformaldehyde/4% sucrose at DIV1, DIV2, and DIV3, respectively. For inhibitor experiments, PLC inhibitor U73122 (0.5 µM; CAYMAN CHEMICAL), MEK/ERK inhibitor U0126 (5 µM; CAYMAN CHEMICAL), PKC inhibitor Sotrastaurin (10 µM; CAYMAN CHEMICAL), or PKC inhibitor Go6983 (1 µM; CAYMAN CHEMICAL) was applied to the culture medium at DIV0, and fixed at DIV3 as described above. The fixed cells were treated with 0.25% Triton X-100 for 5 min, and immunostained with mouse anti-Tuj1 (neuron-specific class III β-tubulin, 1:5000; BioLegend), followed by incubation with donkey Alexa Fluor 488-conjugated anti-mouse IgG antibody (1:500; Thermo Fisher Scientific).

**Image Acquisition And Quantification**

Images of culture experiments were taken with a confocal laser-scanning microscope (TCS SP8; Leica Microsystems) using HC PL APO CS2 20×/0.75 NA multiple immersion lens (Leica Microsystems) or HC PL APO CS 10×/0.40 NA multiple immersion lens (Leica Microsystems) under constant conditions in terms of laser power, pinhole size, gain, z-steps, and zoom setting throughout the experiments. All quantitative measurements were performed with ImageJ 1.52a software [42]. For the quantification of MAP2 and tau staining areas, z-series of optical sections were projected by the brightest point method. MAP2 and tau staining areas were defined as area in which staining signal intensities were 2 and 3 times stronger than those of background signals on the same field, respectively. For analysis of the time course of neurite outgrowth, z-series of optical sections were projected by the brightest point method. Neurite of cortical neurons was identified by Tuj1 signals, and the length of the longest neurite, the number of neurites emerging from the soma, and the numbers of branches per longest neurite were measured.
Western Blot Analysis

Western blot analysis
Primary cortical neuron cultures were prepared as described above and plated on the 24-well dish at a density of $5.0 \times 10^5$ cells/well. One to 100 µM 16:0 LPE or 18:0 LPE was applied to the culture medium at DIV7. After 10 min incubation, the cultures were solubilized with SDS sample buffer containing 2% SDS, 50 mM-Tris-HCl, pH6.8, 10% glycerol, 100 mM Dithiothreitol, phosphatase inhibitor cocktail (Nakarai Tesque), and 0.025% Bromophenol Blue, followed by boiling at 95°C for 10 min. For inhibition of MAPK, 5 µM MEK/ERK inhibitor U0126 was applied to the cell culture medium 30 min before treatment with LPE. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. After blocking with PVDF Blocking Reagent for Can Get Signal (TOYOBO), the membranes were incubated with mouse anti-phospho-MAPK/ERK1/2 (Thr202/Tyr204) antibody (E10, 1:2000; Cell Signaling Technology), followed by incubation with horseradish-peroxidase-conjugated secondary antibody. After stripping, the membranes were probed with rabbit anti p44/42 MAPK antibody (1:1000; Cell Signaling Technology), followed by incubation with horseradish-peroxidase-conjugated secondary antibody. Can Get Signal Immunoreaction Enhancer Solution (TOYOBO) was used for dilution of antibodies. The proteins were visualized by ECL Select Western Blotting Detection System (GE Healthcare) and detected by Las-4000 mini luminescent imaging analyzer (GE Healthcare). The quantification analysis was performed using ImageQuant TL image analysis software (GE Healthcare).

Statistical analysis
Results of at least 2 independent experiments were subjected to statistical analyses. No statistical method was used to determine sample size. No data were excluded. There was no randomization of samples before analysis. Statistical significance was evaluated with two-way or one-way ANOVA followed by Tukey’s, Dunnett’s post hoc test, or Student’s t test, or Kruskal–Wallis test followed by post-hoc Steel–Dwass or Steel’s test using R software (R Core Team, 2017). Statistical significance was assumed when $p < 0.05$.

List Of Abbreviations
CB: cerebrosides, CNS: central nervous system, CL: cardiolipin, DIV: days in vitro, ERK: extracellular signal-regulated kinase, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, MAP2: microtubule-associated protein 2, MAPK: p42/p44 mitogen-activated protein kinase, NeuN: neuronal nuclear antigen, PA: phosphatidic acid, PBS: phosphate-buffered saline, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: Phosphatidylinositol, PKC: protein kinase C, PLC: phospholipase C, PS: phosphatidylserine, PTX: pertussis toxin, 16:0 LPE: palmitoyl LPE, 18:0 LPE: stearoyl LPE

Declarations
Ethics approval and consent to participate
Animal Experiments and approved by the president of Shinshu University (Authorization No. 290072).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

K.H. prepared primary cerebral neurons. K.H. and S.K performed the cell culture experiments. S.K. performed western blot analysis. K.H., S.K., T.M. T.K. and H.Y. performed the quantification analysis. K.H. and T.U. wrote the paper with editing by H.H., T.T., Y.M. and N.S., and T.U. designed and supervised the study. The authors read and approved the final manuscript.

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None

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Figures
Figure 1

Effects of phospholipids on neuronal morphology. a Extracellular application of LPE, PI, or PC increased the MAP2 and tau staining signals in the cultured cortical neurons. The cultures were incubated with 1 μM of indicated phospholipids for 11 days, and immunostained with antibodies against MAP2, tau, and NeuN at DIV14, respectively. Scale bar represents 50 μm. abovine liver LPC; begg yolk LPE; cssoybean PI; dbovine heart SM; eporcine brain PS; fbovine liver PC; gegg yolk PE; hbovine heart CL; ipPA form egg yolk PC; jporcine brain CB. b Quantification of tau signal area in (a). The percentage of tau signal occupied in the observation field was measured. c Quantification of MAP2 signal area in (a). The percentage of tau
signal occupied in in the observation field was measured. d Quantification of number of NeuN positive neurons in (a). The horizontal line in each box indicates the median, the box shows the interquartile range (IQR), and the whiskers are 1.5× IQR. Statistical significance was evaluated with the Kruskal–Wallis test followed by post hoc Steel test: ** p < 0.01 and * p < 0.05, n = 10.

Figure 2

Effects of 16:0 LPE and 18:0 LPE on neuronal morphology. a Extracellular application of 16:0 LPE and 18:0 LPE increased the MAP2 and tau staining signals in the cultured cortical neurons. The cultures were incubated with 0.1, 1 or 10 μM of indicated phospholipids for 11 days, and immunostained with antibodies against MAP2, tau, and NeuN at DIV14, respectively. Scale bar represents 50 μm. b Quantification of tau signal area in (a). The percentage of tau signal occupied in in the observation field was measured. c Quantification of MAP2 signal area in (a). The percentage of MAP2 signal occupied in in the observation field was measured. d Quantification of number of NeuN positive neurons in (a). The horizontal line in each box indicates the median, the box shows the IQR, and the whiskers are 1.5× IQR.
Statistical significance was evaluated with the Kruskal–Wallis test followed by post hoc Steel–Dwass test: ** p < 0.01, n = 10.

Figure 3

Effects of 16:0 LPE and 18:0 LPE on neurite outgrowth. a Extracellular application of 16:0 LPE and 18:0 LPE stimulates neurite outgrowth of the cultured cortical neurons. 16:0 LPE or 18:0 LPE was applied at 1 μM to the cultures at DIV0, and immunostained with antibody against Tuj1 at DIV0, 1, 2, and 3, respectively. Scale bar represents 50 μm. b Quantification of the length of the longest neurite. The longest neurite emerging from the soma was measured in (a). c Quantification of the number of neurites emerging from the soma in (a). d Quantification of the numbers of branches per longest neurite in (a). Black-filled circles, red-filled squares, and blue-filled triangles represent nontreatment control, 16:0 LPE treatment, and 18:0 LPE treatment cultures, respectively. All values represent mean ± s.e.m. Statistical significance was evaluated Kruskal–Wallis test followed by post hoc Steel–Dwass test: *** p < 0.001, n = 60.
Figure 4

Dose-dependent stimulation of neurite outgrowth by application of 16:0 LPE and 18:0 LPE. a Application of 16:0 LPE and 18:0 LPE stimulates neurite outgrowth of cultured cortical neurons in a dose dependent manner. Indicated concentration of 16:0 LPE or 18:0 LPE was applied to the cultures at DIV0, and immunostained with antibody against Tuj1 at DIV3, respectively. Scale bar represents 50 μm. b Quantification of the length of the longest neurite in (a). Red-filled squares and blue-filled triangles represent 16:0 LPE-treatment and 18:0 LPE-treatment cultures, respectively. All values represent mean ± s.e.m. Statistical significance was evaluated using two-way ANOVA, n = 20.
Figure 5

Effects of PLC and PKC inhibitors on 16:0 LPE- and 18:0 LPE-stimulated neurite outgrowth. a Different effect of PLC inhibitor U73122 on 16:0 LPE- and 18:0 LPE-stimulated neurite outgrowth. b PKC inhibitor Go6983 inhibits 18:0 LPE-stimulated neurite outgrowth but not 16:0 LPE-stimulate neurite outgrowth. c PKC inhibitor Sotrastaurin inhibits 16:0 LPE- and 18:0 LPE-stimulated neurite outgrowth. 16:0 LPE or 18:0 LPE at 1 μM was applied to the cultures with or without 0.5 μM U73122 in (a), 1 μM Go6983 in (b), or 10 μM Sotrastaurin in (c) at DIV0, and immunostained with antibody against TuJ1 at DIV3, respectively. d–f Quantification of the length of the longest neurite in (a–c). Scale bars represent 50 μm in (a–c). The horizontal line in each box indicates the median, the box shows the IQR, and the whiskers are 1.5× IQR. Statistical significance was evaluated with the Kruskal–Wallis test followed by post hoc Steel–Dwass test: *** p < 0.001, ** p < 0.01, * p < 0.05, n = 60.
Figure 6

Effects of 16:0 and 18:0 LPE on activation of MAPK in cultured cortical neurons. a 16:0 LPE and 18:0 LPE induce the phosphorylation of MAPK/ERK1/2. At DIV7, the cultures were incubated with indicated concentrations of 16:0 LPE or 18:0 LPE for 10 min. Lysates were subjected to SDS-PAGE, followed by Western blot analysis using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. b Quantification of phosphorylated ERK1/2 signals in (a). Intensities of phosphorylated ERK1/2 signals were normalized
with those of ERK1/2 signals. Red-filled squares and blue-filled triangles represent 16:0 LPE-treatment and 18:0 LPE-treatment cultures, respectively. All values represent mean ± s.e.m. Statistical significance was evaluated using two-way ANOVA followed by post hoc Student’s t test. n = 3.

Figure 7

Effects of MAPK inhibitor U0126 on 16:0 LPE- and 18:0 LPE-stimulated neurite outgrowth. a U0126 inhibits 16:0 LPE or 18:0 LPE-induced phosphorylation of ERK1/2. At DIV7, the cultures were incubated with 100 μM 16:0 LPE or 18:0 LPE, with or without 5 μM U0126 for 10 min. Lysates were subjected to SDS-PAGE, followed by Western blot analysis using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. b Quantification of phosphorylated ERK1/2 signals in (a). Intensities of phosphorylated ERK1/2 signals were normalized with those of ERK1/2 signals. All values represent mean ± s.e.m. c MAPK inhibitor U0126 inhibited 18:0 LPE-stimulated neurite outgrowth and partially inhibited 16:0 LPE-stimulated neurite outgrowth. 16:0 LPE or 18:0 LPE at 1 μM was applied to the cultures with or without 5 μM U0126 at DIV0,
and immunostained with antibody against Tuj1 at DIV3. Scale bar represents 50 μm. d Quantification of the length of the longest neurite in (c). The horizontal line in each box indicates the median, the box shows the IQR, and the whiskers are 1.5× IQR. Statistical significance was evaluated with the Kruskal–Wallis test followed by post hoc Steel–Dwass test in (b) or evaluated using one-way ANOVA followed by post hoc Tukey’s test in (d). *** p < 0.001, ** p < 0.01, and * p < 0.05, n = 60 in (b) and n = 3 in (d), respectively.