Lysophosphatidylethanolamine in *Grifola frondosa* as a neurotrophic activator via activation of MAPK

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Abstract **We found that *Grifola frondosa* extracts induced the activation of mitogen-activated protein kinase (MAPK) in cultured PC12 cells, a line of rat pheochromocytoma cells. The active substance was isolated by a few chromatographic steps, including high-performance liquid chromatography, and was identified to be lysophosphatidylethanolamine (LPE) from various structural analyses. LPE from *G. frondosa* (GLPE) was confirmed to induce the activation of MAPK of cultured PC12 cells and was found to suppress cell condensation and DNA ladder generation evoked by serum deprivation, suggesting that the GLPE had antiapoptotic effects. Moreover, GLPE caused morphological changes in and upregulation of neurofilament M expression of PC12 cells, demonstrating that the GLPE could induce neuronal differentiation of these cells. The activation of MAPK by GLPE was suppressed by AG1478, an antagonist of epidermal growth factor receptor (EGFR), and by U0126, an inhibitor of MAPK kinase (MEK1/2), but not by K252a, an inhibitor of TrkA, or by pertussis toxin. These results demonstrate that GLPE induced the MAPK cascade [EGFR-MEK1/2-extracellular signal-regulated protein kinases (ERK1/2)] of PC12 cells, the activation of which induced neuronal differentiation and suppressed serum deprivation-induced apoptosis. This study has clarified for the first time the involvement of the MAPK signal cascade in LPE actions.**—Nishina, A., H. Kimura, A. Sekiguchi, R-h. Fukumoto, S. Nakajima, and S. Furukawa. **Lysophosphatidylethanolamine in *Grifola frondosa* as a neurotrophic activator via activation of MAPK. *J. Lipid Res.* 2006.** 47: **1434–1443.**

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Natural compounds with antioxidant activity, such as curcumin (1), phenolic yellow curry pigment, and naringenin, a major flavonone constituent isolated from *Citrus junos*, have been found to reduce the neurotoxicity generated by amyloid β protein and to result in reduced amyloid deposition and the amelioration of drug-induced amnesia in animal models of Alzheimer's disease. Soy isoflavones have similarly been shown to influence the brain cholinergic system, reducing age-related neuron loss and the spatial cognition decline that occurs in elderly rats (2). These observations prompted us to search for natural compounds with neurotrophic and/or neuroprotective activities, because the population of the Western world is progressively aging, and this increase in the elderly population will mean an increase in age-related cognitive decline disorders such as Alzheimer's disease. Recent investigations suggest that neurotrophic factors are involved in the cause and/or development of these diseases and that the development of compounds with activities like those of particular neurotrophic factors may be a promising avenue for protection against such diseases (3–5).

Although many edible fungi are cultivated all over the world, we focused on mushrooms, because they are marketed as healthful foods and their pharmacological activities have been studied. Tricholomalides from *Tricholoma* species (6), termitomycesphins from *Termitomyces albuminosus* (7), and dictyoquinazol and dictyophorines from *Dictyophora indusiata* (8, 9) have been found to be active toward neurons. The biological activities of various mushroom components have been studied. For example, the exopolysaccharide from the culture broth of *Hericium erinaceus* was shown to enhance the growth and neuronal differentiation of PC12 cells (10). The apoptosis of PC12 cells was evoked by the culture broth of *Lentinula edodes* (11), and the apoptosis of U937 cells was evoked by a lectin isolated from the mushroom *Boletopsis leucomelas* (12).

Grifola frondosa (Maitake mushroom), one of the most widely grown mushrooms in Gunma Prefecture of Japan,

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was selected as the source for isolation of active substances in this study because we were particularly interested in the activity of natural components toward neuronal cells. We evaluated the activity of mushroom extracts for the induction of phosphorylation of mitogen-activated protein kinase (MAPK) in cultured rat pheochromocytoma PC12 cells, because the Raf (MAPK kinase kinase)/MEK1/2 (MAPK kinases)/extracellular signal-regulated protein kinases (ERK1/2) (MAPKs, extracellular signal-regulated protein kinases) pathway has been well studied, and ERK1/2 activity serves as one of the checkpoints controlling cellular differentiation and proliferation. As a result, we identified lysophosphatidylethanolamine (LPE) as an active substance that induced the phosphorylation of ERK1/2, the activation of which was followed by inhibition of serum deprivation-induced apoptosis and induction of neuronal differentiation in cultured PC12 cells.

MATERIALS AND METHODS

Reagents

G. frondosa (Maitake mushroom) powder was a gift from Yukiguni Maitake Co., Ltd. (Niigata, Japan). Ethyl acetate, *n*-hexane, chloroform, methanol, acetic acid, NaF, NaCl, and sodium deoxycholate were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). PMSF, Nonidet P-40, aprotinin, and agarose (type I, low electroendsosis) were purchased from Sigma (St. Louis, MO). BSA was obtained from Serologicals Corp. (Norcross, GA). SDS, Na_3VO_4, leupeptin, and an inhibitor for the Trk-type tyrosine kinase (K252a) were purchased from Wako Pure Chemical Industries (Osaka, Japan). RNase and a DNA size marker (100 bp ladder) came from MoBiTec (Goettingen, Germany) and Promega Corp. (Madison, WI), respectively. MEK inhibitors (PD98059 and U0126) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Specific inhibitors for epidermal growth factor receptor (EGFR) tyrosine kinase (AG1478) and a Gi/o protein pertussis toxin (PTX) were from Calbiochem (San Diego, CA) and List Biological Laboratories, Inc. (Campbell, CA), respectively.

Preparation of the active component from *G. frondosa*

Powdered *G. frondosa* (100 g) was immersed in 500 ml of a chloroform-methanol mixture (2:1, v/v) for 24 h at room temperature. The solvent containing the extracts were filtered through a filter paper (5C; Whatman, Blentford, UK), and the filtrate was evaporated to dryness. The solid extract (2.0 g) was then stirred in 10 ml of *n*-hexane at room temperature for 1 h, after which the mixture was centrifuged at 1,500 *g* at room temperature for 10 min. The supernatant was collected and evaporated to dryness. The sample was suspended in 10 ml of ethyl acetate, and the precipitate was then sequentially extracted, first with chloroform and then with methanol. The material that was obtained after the methanol extract had been evaporated to dryness was called the residue. The fraction that remained insoluble after each extraction was dissolved in chloroform-methanol (2:1, v/v) at a concentration of 100 mg/ml. An aliquot of the chloroform-methanol solution was injected into a HPLC apparatus equipped with a Develosil 60-10 (for normal phase) or Develosil ODS 60-10 (for reverse phase) column (20 × 250 mm, 10 μm particle size; Nomura Chemical Co., Ltd., Aichi, Japan), a PU-800 pump (JASCO, Tokyo, Japan), and an evaporative light-scattering detector (SEDEX model 75; Sedere, Inc., Cranbury, NJ). The column was run with hexane-ethyl acetate (80:20, v/v) for the normal phase or methanol for the reverse phase at a flow rate of 10 ml/min. Eluted substances were manually collected by referring to the signal detected by the evaporative light-scattering detector and subjected to TLC analysis using silica-gel 60 (Merck KgaA) with a developing solvent of chloroform-methanol-acetic acid-water (25:15:4:2, v/v/v/v). Detection was performed using sulfuric acid for total organisms, anthrone reagent for glycolipids, iron chloride reagent for sterols, Ditter's reagent for phosphate, and ninhydrin reagent for amino bases. Components of each detection reagent were reported previously (13–15).

Cell culture and bioassay

PC12 cells were cultured as described previously (16). In brief, the cells were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated horse serum (Gibco BRL, Grand Island, NY) and 5% heat-inactivated FBS (Sanko Junyaku, Co., Ltd., Tokyo, Japan) or in serum-free medium (DMEM supplemented with 1% BSA), unless stated otherwise.

All *G. frondosa* samples, such as the chloroform-methanol extract, fractions obtained by HPLC, and reagents, were prepared in serum-free DMEM and sonicated until fully emulsified. PC12 cells were seeded at a cell density of 2×10^6 cells/well into collagen-coated six-well plates containing medium with serum and precultured for 2 days at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were then washed with PBS and incubated with the above-mentioned culture medium containing a given sample from *G. frondosa* or various agents for 10 min at 37°C. Then, the culture plates were placed on ice, and each well was washed with 3 ml of 2 mM TBS containing 0.33 M NaF and 6.25 M Na_3VO_4 and subsequently lysed with 150 μl of 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (w/v), 1% sodium deoxycholate (w/v), 0.1% SDS (w/v), 50 mM NaF, 0.1% aprotinin (w/v), 0.1% leupeptin (w/v), 1 mM Na_3VO_4, and 1 mM PMSF. Cell lysates were collected using a cell scraper, transferred to 1.5 ml microcentrifuge tubes, and centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was collected and transferred to another tube, and the overall protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with BSA as the standard.

Analysis of the chemical structure of the active substance for MAPK activation

HPLC-time of flight MS was performed using a micro-time of flight focus (Bruker Daltonik GmbH, Bremen, Germany) equipped with a Develosil ODS 60-5 column (4.5 × 250 mm, 5 μm particle size; Nomura Chemical), and the active components were eluted by methanol. Infrared spectra were recorded with a Nicolet Magna-500 infrared spectrometer. ^{1}H-NMR spectra were recorded with a JEOL AL-300 spectrometer, with chemical shifts being reported on the δ scale in ppm relative to Me_4Si. ^{13}C-NMR, ^{1}H-^{1}H total correlation spectroscopy, ^{1}H-^{13}C heteronuclear single quantum correlation, and ^{1}H-^{13}C ^{1}H-detected heteronuclear multiple bond connectivity spectra were recorded with a Bruker AV-400 spectrometer.

Detection of phosphorylated proteins

Proteins (20 μg) in each supernatant were mixed with SDS sample buffer and incubated for 5 min at 80°C. Protein samples were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride filters (Fluorotrans membrane W, 0.2 μm; Nihon Genetics, Tokyo, Japan). Immunoblotting analysis was performed using monoclonal antibodies against p44/42

ERK, phospho p44/42 ERK (Cell Signaling Technology, Lake Placid, NY), and neurofilament M (NF-M; Sigma) as primary antibodies, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega) as the secondary antibody. The blots were developed by the enhanced chemiluminescence method (Hyperfilm-ECL plus; Amersham Biosciences Corp., Piscataway, NJ).

Treatment with specific inhibitors of signal transduction

Each inhibitor was added to serum-containing medium to result in a final concentration of 1 μM (K252a, PTX, and AG1478), 50 μM (U0126), or 200 μM (PD98059). Cells were preincubated with each inhibitor for 20 h (PTX) or 4 h (all others) and cultured in the serum-containing medium supplemented with each extract or reagent for the appropriate times. The cells were then collected and subjected to analysis of phosphorylation or expression of proteins as described above. Cytotoxicity of U0126 or PD98059 was measured by MTT assay (17).

Analysis of apoptotic DNA fragmentation

PC12 cells maintained for 2 days on six-well plates (2×10^6 cells/well) at 37°C in an atmosphere containing 5% CO_2 were washed with PBS and incubated in serum-free medium for 0, 1, 2, or 8 days. Then, the cells were collected and centrifuged at 500 *g* for 5 min. DNA was extracted from the cells using a QIAamp DNA mini kit (Qiagen, Valencia, CA). Fragmentation of DNA was confirmed on 3% agarose gels after electrophoresis and staining with ethidium bromide for visualization under ultraviolet transillumination.

NF-M expression in PC12 cells

PC12 cells plated on poly-D-lysine-coated cover glasses (13 × 13 mm; Matsunami Glass Industries, Ltd., Osaka, Japan) were precultured in serum-containing medium for 24 h, washed with PBS, and incubated with the same medium containing mushroom extract or agent for the appropriate period. Cells were fixed with paraformaldehyde solution (4%, w/v) and incubated for 20 min in PBS containing Triton X-100 (0.3%, v/v). Nonspecific binding was blocked with Block Ace solution (2%, v/v; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Cells were treated with anti-NF-M monoclonal antibody (Sigma) and then reacted with Alexa Fluor 488-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR). The stained samples were mounted on slide glasses by use of Tissue-Tek (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and observed with a confocal laser scanning microscope (Radiance 2100; Bio-Rad, Hercules, CA).

RESULTS

MAPK activation of PC12 cells by *G. frondosa* extracts

Activation of ERK1/2 is one of the checkpoints to assess the activation of the classical Ras/MAPK cascade (18), which is triggered by an engaged tyrosine kinase receptor or G protein-coupled receptor and results in proliferation and/or differentiation. Therefore, we tested whether the *G. frondosa* contained something that could activate this signal pathway. The whole extract obtained from 100 g of dried *G. frondosa* was fractionated with various solvents, and the extracts obtained by use of *n*-hexane (1.39 g), ethyl acetate (0.04 g), chloroform (0.02 g), and methanol (0.34 g) as well as the residue (0.11 g) were evaluated for their ability to induce the activation of ERK1/2 (**Fig. 1A**).

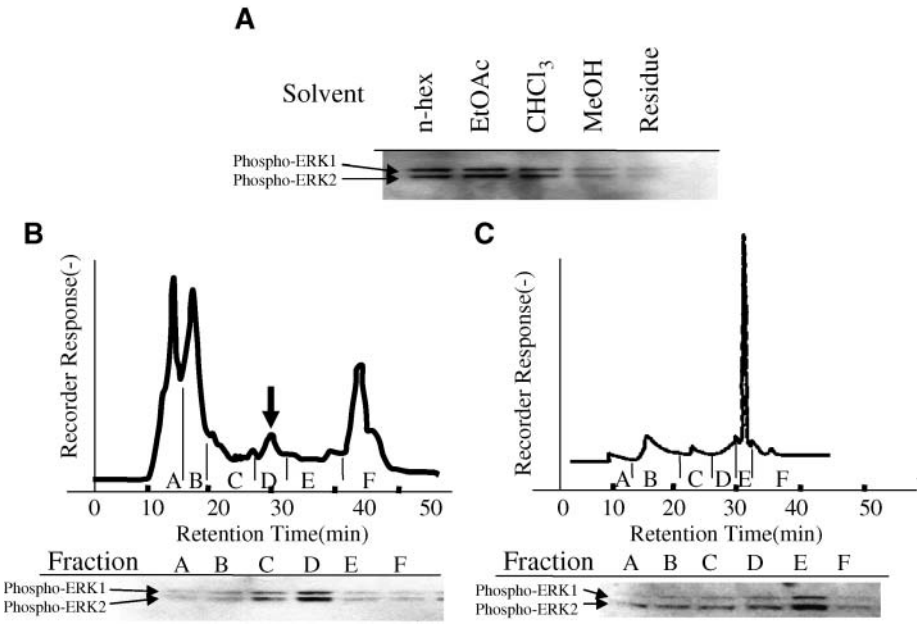


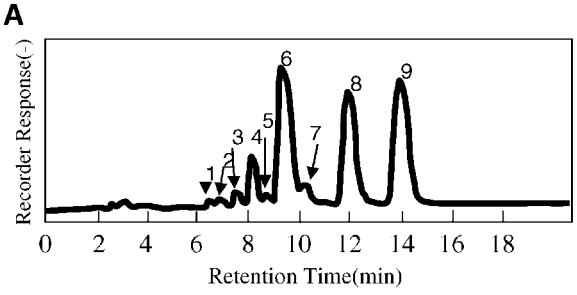
Fig. 1. Extraction and fractionation of the components of *G. frondosa* and the ability of each fraction to activate extracellular signal-regulated protein kinases (ERK1/2). A: Effects of the fractions extracted by various organic solvents on the activation of ERK1/2. n-hex, EtOAc, $CHCl_3$, and MeOH indicate the solvents *n*-hexane, ethyl acetate, chloroform, and methanol, respectively. B: Typical chromatographic profile of *n*-hexane extracts by HPLC with a silica-gel column, and the distribution of mitogen-activated protein kinase activation activities. C: Chromatographic pattern of the active fraction D obtained from panel B by HPLC using an ODS column, and the distribution of the activities.

The extracts obtained with low-polarity solvents showed stronger activity, as the amounts of the *n*-hexane and ethyl acetate extracts were 70 and 35 times, respectively, greater than those in the chloroform extract. Therefore, we chose the *n*-hexane extract as the starting material for the isolation of the putative active component. At first, the *n*-hexane extract was fractionated by preparative normal-phase HPLC, and the fraction with potent activity (indicated by the arrow in Fig. 1B) was subjected to reverse-phase HPLC (resulting in fraction E in Fig. 1C). Fraction E of the second HPLC with the activity was further analyzed by TLC. After development, the TLC plate was stained with sulfuric acid (for detection of all organic compounds), Dittmer's reagent (for detection of phosphorus compounds), or ninhydrin (for detection of amino-containing compounds). The results showed that the active component was an amino group-containing phospholipid. The relative mobility value of the active components was in good agreement with that of LPE (data not shown). Infrared spectra of the active component showed the characteristic peak at 1,730.5 cm^{-1} for carbonyl stretching of the ester as well as a broad band at ~3,000 cm^{-1} attributable to the primary amine salt (N-H stretching) along with C-H stretching peaks of alkanes. ^{1}H-NMR spectra of the active component showed signals at δ = 3.08 ppm (2H) and 3.98 ppm (2H; protons of two methylenes of ethanolamine moiety) and δ = 3.82 ppm (2H), 3.90 ppm (1H), and 4.06 ppm (2H; protons of the glycerol moiety). ^{13}C-NMR peaks of four carbons (both of the ethanolamine carbons and two of the three glycerol carbons) were split in two as a result of the spin coupling with ^{31}P. ^{1}H-NMR, infrared analysis, and mass spectra of synthetic 1-myristoyl-LPE, 1-palmitoyl-LPE, and 1-oleoyl-LPE were similar to those of the active component from *G. frondosa* (data not shown). As shown in **Fig. 2**, the tentative LPE concentrations calculated from the area of the total ion chromatogram were 0.8% for 1-myristoyl-LPE, 1.9% for 1-margaroyl-LPE, 4.1% for 1-palmitoyl-LPE, 3.2% for 1-palmitoleoyl-LPE, 39.1% for 1-oleoyl-LPE, 49.3% for 1-linoleoyl-LPE, and 1.6% for 1-linolenoyl-LPE. These results confirmed that the compound from *G. frondosa* that induced ERK1/2 activation in PC12 cells was LPE. Moreover, synthetic 1-myristoyl-LPE, 1-palmitoyl-LPE, and 1-oleoyl-LPE activated ERK1/2 of PC12 cells equally to GLPE (**Fig. 3**).

Characterization of GLPE

GLPE was further fractionated by reverse-phase HPLC, and 90.8% of 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine obtained was used for the characterization of GLPE. Next, the effect of GLPE on the phosphorylation of ERK1/2 was examined, and we found that GLPE induced the phosphorylation of ERK1/2 in a dose-dependent manner from 200 to 2,000 μM (Fig. 3). It is known that nerve growth factor (NGF) induces the activation of MEK1/2, a MAPK kinase, and phosphorylates ERK1/2 (19). Therefore, the effects of MEK inhibitors (U0126 and PD98059) on the induction of phosphorylation of ERK1/2 by NGF or GLPE were evaluated (**Fig. 4**). The induction of activation of ERK1/2 by both NGF and GLPE was similarly inhibited by both inhibitors; that is, it was suppressed greatly by U0126 and slightly by PD98059. Therefore, it was obvious that GLPE, as well as NGF, induces the activation of MEK1/2, resulting in the activation/phosphorylation of ERK1/2.

The cytotoxicity of PD98059 or U0126 for PC12 cells was checked by MTT assay, because they were used at relatively high concentrations in this study. No toxicity was detected, even at the highest concentration shown in Fig. 4 (data not shown).



B

Fraction No.	Measured MW	Estimated Structural Formula	Theoretical MW
1	426.26	C19H41N1O7P1=LPE(14:0)	426.262
2	476.275	C23H43N1O7P1=LPE(18:3)	476.277
3	452.276	C21H40N1O7P1=LPE(16:1)	452.277
4	478.292	C23H45N1O7P1=LPE(18:2)	478.293
5	440.277	C20H43N1O7P1=LPE(15:0)	440.277
6	478.292	C23H45N1O7P1=LPE(18:2)	478.293
7	454.292	C21H45N1O7P1=LPE(16:0)	454.293
8	454.293	C21H45N1O7P1=LPE(16:0)	454.293
9	480.309	C23H47N1O7P1=LPE(18:1)	480.308

C

Fatty acid Bonded to LPE	Concentration(%) Estimated from Area in TIC
C14:0	0.8
C15:0	1.9
C16:0	4.1
C16:1	3.2
C18:1	39.1
C18:2	49.3
C18:3	1.6
total	100

Fig. 2. Confirmation of fatty acids bound to lysophosphatidylethanolamine (LPE) obtained from *G. frondosa*. A: Pattern of total ion chromatography of fraction E shown in Fig. 1C. B: Exact mass measurements of peaks obtained by total ion chromatography. MW, molecular weight. C: Composition of fatty acids bound to LPE of *G. frondosa* (GLPE) calculated from peak areas on the total ion chromatogram (TIC).

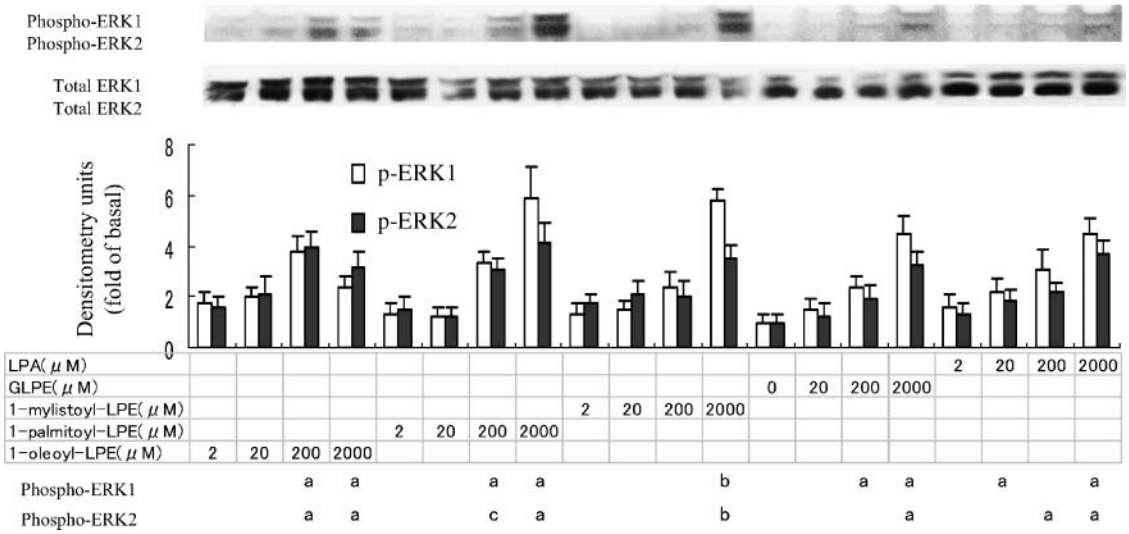


Fig. 3. Concentration-dependent phosphorylation of ERK1/2 of PC12 cells by GLPE, synthetic LPE, and lysophosphatidic acid (LPA). The concentrations indicated for each sample are final concentrations in the culture medium. Cells were treated with each sample for 30 min, and the amounts of phosphorylated ERK1/2 were visualized by Western immunoblotting. Data are normalized to the level of total ERK and expressed as means ± SEM of three separate experiments. Significant differences from the value of the corresponding control group were determined by Student's *t*-test and are indicated at bottom as follows: a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$.

Effects of inhibitors of NGF or EGFR kinase, or an inhibitor of $G_{i/o}$, on the phosphorylation of ERK1/2

The effects of K252a (an inhibitor of the tyrosine kinase of the high-affinity NGF receptor Trk) and AG1478 (an inhibitor of the tyrosine kinase of the EGFR) on GLPE-, NGF-, and EGF-induced phosphorylation of ERK1/2 of PC12 cells were examined next, as was the effect of PTX (one of the $G_{i/o}$ inhibitors) on GLPE-induced phosphorylation (**Fig. 5**). K252a and AG1478 suppressed the induction of phosphorylation by NGF and EGF, respectively. The phosphorylation of ERK1/2 induced by GLPE was suppressed by AG1478, but not by K252a or PTX, suggesting that GLPE induced the phosphorylation of ERK1/2 via activation of the EGFR tyrosine kinase but not via the NGF receptor or PTX-sensitive G protein-coupled receptor (GPCR) ($G_{i/o}$).

Protective effects of GLPE against serum deprivation-induced apoptosis of PC12 cells

As NGF is known to prevent PC12 cells from undergoing apoptosis induced by serum deprivation (20), such activity of GLPE was investigated. PC12 cells were plated on collagen-coated 12-multiwell plates at a cell density of 10^4/well and cultured for 2 days in medium supplemented with 10% horse serum and 5% FBS. The medium was then exchanged for serum-free medium containing 1% BSA and various concentrations of GLPE or NGF, and the cells were cultured for another 2 days. A substantial number of cells underwent apoptosis when the cells were cultured in control medium containing neither NGF nor GLPE, as judged from the cell body condensation and DNA ladder formation (**Fig. 6**). However, most PC12 cells survived and generated neurites in the presence of NGF or GLPE. These

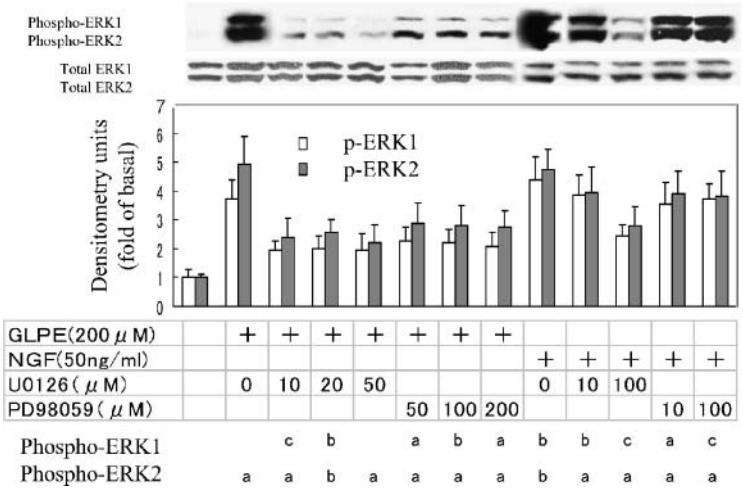


Fig. 4. Effects of MAPK kinase (MEK1/2) inhibitors on ERK1/2 activation by GLPE or NGF. PC12 cells were treated with U0120 or PD98059 as described in the text. Cells were collected after treatment with NGF (50 ng/ml) or GLPE (200 μM) for 10 min, and ERK1/2 activation was evaluated by immunoblotting. Data are normalized to the level of total ERK and expressed as means ± SEM of three separate experiments. Significant differences from the value of the corresponding control group were determined by Student's *t*-test and are indicated at bottom as follows: a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$.

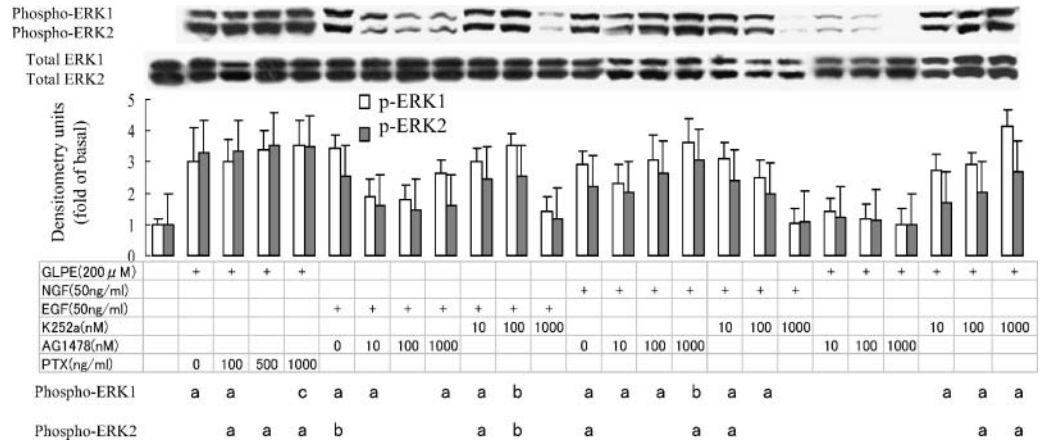


Fig. 5. Effects of receptor kinase inhibitors on ERK1/2 activation by GLPE, EGF, or NGF. PC12 cells were treated with K252a (10–1,000 nM), AG1478 (10–1,000 nM), or pertussis toxin (100–1,000 ng/ml) as described in the text. Cells were collected after treatment with NGF (50 ng/ml), EGF (50 ng/ml), or GLPE (200 μM) for 10 min, and ERK1/2 activation was evaluated by immunoblotting. Data are normalized to the level of total ERK and expressed as means ± SEM of three separate experiments. Significant differences from the value of the corresponding control group were determined by Student's *t*-test and are indicated at bottom as follows: a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$.

results demonstrate that GLPE suppressed the apoptosis of PC12 cells induced by serum deprivation as efficiently as NGF.

Neuronal differentiation of PC12 cells by GLPE

PC12 cells were plated on poly-D-lysine-coated 12-multiwell plates at a cell density of 2×10^6 or 1×10^4 cells/well for immunoblot analysis or morphological observation, respectively, and cultured for 2 days in the presence of NGF or EGF at 50 ng/ml or GLPE at 200–1,000 μM. Expression of NF-M protein of each culture was analyzed by immunostaining of the cells (**Fig. 7**) and by Western immunoblotting (**Fig. 8**). From the results of the immuostaining, the expression intensity of NF-M protein and the percentage of NF-M-positive cells increased when the cells vigorously extended neurites 5 days after the addition of NGF. However, neither EGF nor vehicle treatment (control) had any effects. GLPE significantly increased NF-M expression in PC12 cells at 2 days, but the expression be-

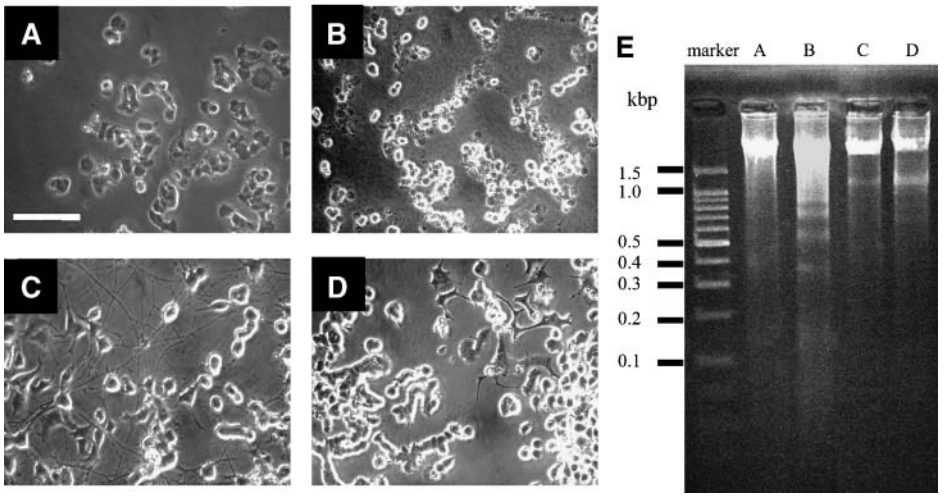


Fig. 6. Serum deprivation-induced apoptosis of PC12 cells and its suppression by GLPE or NGF. A: PC12 cells were cultured in serum-containing medium for 2 days. B–D: The medium was then changed to serum-free medium not containing (B) or containing NGF (50 ng/ml; C) or GLPE (200 μM; D), and the cells were cultured for another 2 days. Photographs were taken under phase-contrast observation at 100× magnification. Bar = 10 μm. E: Cells were cultured in serum-free medium for 0 or 48 h, and DNA fragmentation was measured by electrophoresis. Columns A, B, C, and D in E correspond to panels A, B, C, and D, respectively. Marker indicates size markers.

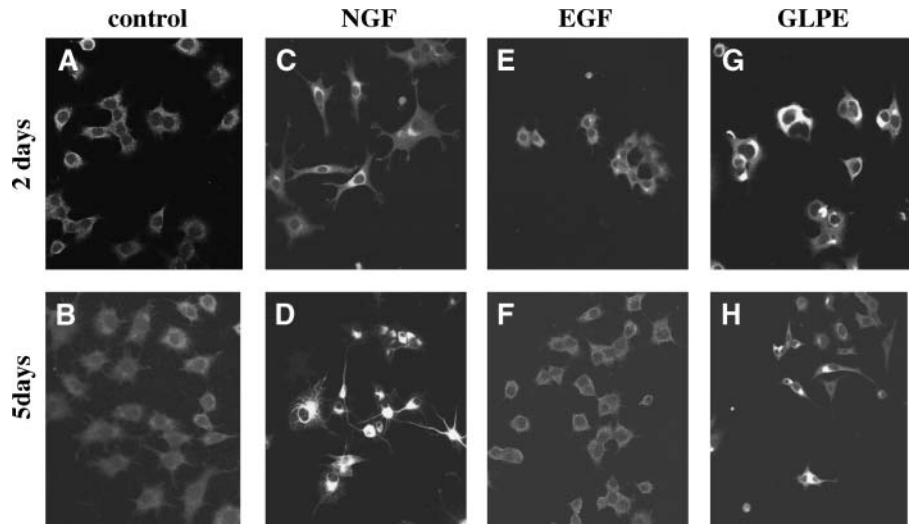


Fig. 7. Expression of neurofilament M (NF-M) in PC12 cells cultured in the presence of GLPE, EGF, or NGF. PC12 cells were plated on poly-D-lysine-coated 12-multiwell plates at 10^4 cells/well and cultured for 2 (A, C, E, G) or 5 (B, D, F, H) days in medium supplemented with NGF (50 ng/ml; C, D), EGF (50 ng/ml; E, F), or GLPE (200 μM; G, H) or without any agent (control; A, B). Cells fixed with 4% paraformaldehyde solution were immunostained with anti-NF-M antibody followed by Alexa Fluor 488-conjugated anti-rabbit antibodies and photographed at 600× magnification with a camera attached to a confocal laser scanning microscope.

came reduced by 5 days. Neurite outgrowth by GLPE was weaker than that by NGF.

The results of the immunoblot analysis (Fig. 8) revealed that the addition of NGF increased the expression level of NF-M protein but that EGF or vehicle (control) treatment was not efficient. On the other hand, different from the immunostaining results, GLPE at 1,000 μM was necessary for detectable expression of NF-M. Comparing the results of Figs. 7, 8, we suspect that cell density might affect the efficient dose of GLPE for NF-M expression.

Effects of MEK1/2 inhibitor on neurite outgrowth and expression of NF-M

NF-M expression induced by NGF (Fig. 8B) and neurite outgrowth (**Fig. 9**) were markedly inhibited by U0126. Similarly, the neurite outgrowth and NF-M expression induced by GLPE were obstructed by U0126. From these results, we propose that GLPE induced neurotrophic effects on PC12 cells via induction of MEK1/2 activation, because phosphorylation of ERK1/2 in PC12 cells is known to be caused predominantly by the activation of MEK1/2 (21).

The results described above show that GLPE elicited the phosphorylation of ERK1/2 via the EGFR (Fig. 5) but weakly affected the proliferation of PC12 cells in a way different from EGF. Additionally, GLPE had neurotrophic effects (i.e., it induced neuronal differentiation of PC12 cells like NGF). However, the induction of NF-M expression and neurite outgrowth by GLPE was weaker than that by NGF.

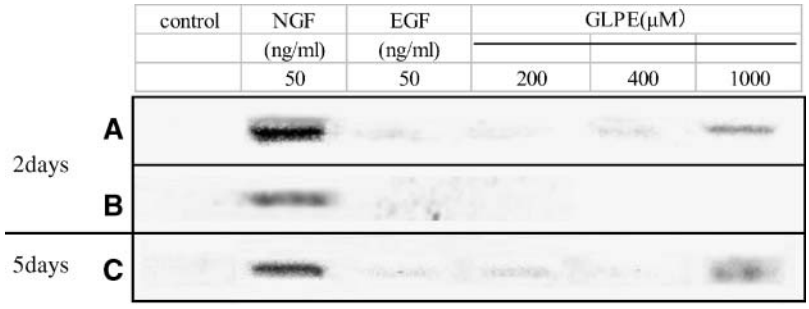


Fig. 8. Expression of NF-M in PC12 cells in serum-containing medium supplemented with U0126, GLPE, EGF, or NGF. PC12 cells were plated on poly-D-lysine-coated six-multiwell plates at 10^4 cells/well and cultured in serum-containing medium for 2 days. The medium was then changed to serum-containing medium supplemented with (B) or without (A, C) U0126 (50 nM) and NGF (50 ng/ml), EGF (50 ng/ml), or GLPE (200–1,000 μM), and the cells were cultured for another 2 or 5 days. The cells were collected, and the amount of NF-M expression was measured by immunoblotting.

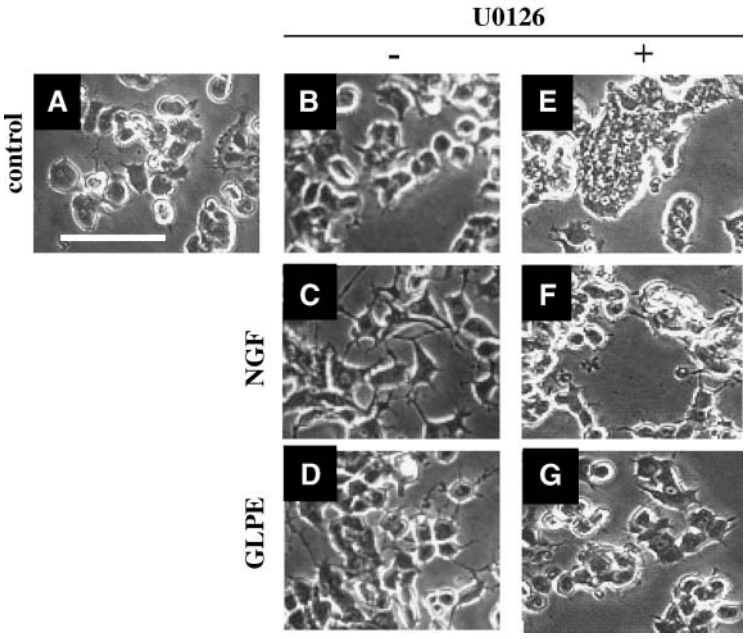


Fig. 9. Effects of MEK1/2 inhibitor on GLPE- or NGF-induced morphological changes. A: PC12 cells were cultured in serum-containing medium for 2 days. B–G: The medium was then changed to serum-containing medium supplemented with U0126 (50 nM; E–G) and NGF (50 ng/ml; C, F) or GLPE (200 μM; D, G), and the cells were incubated for another 2 days. Photographs were taken with a phase-contrast microscope at 100× magnification. Bar = 10 μm.

DISCUSSION

A well-known neurotrophic factor, NGF, acts on cultured PC12 cells and induces many processes, including neurite outgrowth for their neuronal differentiation into sympathetic neuron-like cells. These NGF actions require both phosphorylation of the NGF receptor, TrkA, expressed on the cell surface, as a trigger and subsequent activation of the MAPK cascade (22). Based on these facts, extracts of *G. frondosa* were tested and found to induce ERK1/2 phosphorylation of PC12 cells as efficiently as NGF. An active component was isolated from the extracts and identified as LPE. To date, the pharmacological effects of LPE are unclear; although phosphatidylethanolamine is a common cell membrane component, and LPE is easily derived from phosphatidylethanolamine by deacylation by phospholipase A_2.

The LPE concentration necessary to induce the activation of ERK1/2 of PC12 cells was almost 200 μM in our assay system. On the other hand, Howe and Marshall (23) demonstrated that the concentration of lysophosphatidic acid (LPA), an analog of LPE, necessary to induce the activation of ERK1/2 of PC12 cells was <200 nM. In this study, the effect of 1-oleoyl-LPE on ERK1/2 activation was greater than that of an equal concentration of 1-oleoyl-LPA (Fig. 3). As phospholipids such as LPA and LPE have polar and nonpolar groups in their structures, they can be precipitated or emulsified by addition to aqueous medium. In recent studies, various solvents or emulsifiers such as DMSO and methanol were used to dissolve phospholipids into aqueous solution, which may cause serious problems for estimation of the activities of these phospholipids. Therefore, in this study, LPE was added to the medium by supersonic dispersion (24), using neither solvents nor emulsifier.

We confirmed by the following methods that LPE was the single active component contained in GLPE. First, NMR analysis of GLPE gave no signals other than those of LPE. Two kinds of TLC conditions using silica-gel plates or ODS plates developed with chloroform-methanol-water (120:70:2) or chloroform-acetic acid-water (46:25:2.5) were used to check for impurities with the same relative mobility value as LPE. From the results mentioned above, even if a compound other than LPE was contained in the GLPE sample, it would seem to be less important. It is necessary to compare the activity of the phospholipids with consideration of their solubility in the near future.

Phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine are major phospholipids of the plasma membrane of mammalian cells; phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol are major phospholipids in the plant kingdom (25). Pharmacological studies on phosphatidylethanolamine demonstrated suppression of MAPK activation (26), Raf-1 kinase inhibition (27), induction of human keratinocyte differentiation (28), and activation of heterotrimeric G protein (29).

LPE is a phosphatidylethanolamine molecule lacking a fatty acid. Although the induction of transformin growth factor-β release from the extracellular matrix of chondrocytes by LPE was reported recently (30), the function of LPE has not yet been fully clarified. To our knowledge, this study is the first to show the antiapoptotic activity and neuronal differentiation-stimulating property of LPE. Among the lysophospholipids, platelet-agglutinating factor (31), lysophosphatidylcholine (LPC) (32), and LPA (33–36) have been investigated for their pharmacological activities. It is noteworthy that LPA activates MAPK via GPCR (37) or induces neurite retraction in PC12 cells (38, 39). This study has shown that LPE, the functional group of which is different from that of LPA, activated MAPK of PC12 cells via the EGFR and facilitated neurite outgrowth. Of interest, LPE induced the activation of the MEK-ERK pathway via the EGFR, not through the NGF receptor

(Fig. 5). However, the effects of LPE on PC12 cells were neuroprotective and/or neurotrophic, like those of NGF and different from those of EGF (Figs. 7, 8).

G_2A and $S1P_{1-3}$ have been identified as receptors of LPC and sphingosine-1-phosphate (S1P), respectively (40–42). G_2A is activated not only by LPC but also by LPA (43). Neurite outgrowth is enhanced by the activation of $S1P_1$ as part of a chain reaction that is triggered by the activation of TrkA by NGF and suppressed by the activation of $S1P_2$ that accompanies cell differentiation (41). As the mechanism of MAPK phosphorylation induced by LPE, direct stimulation by LPE through its unknown receptor(s) or indirect stimulation by LPA or LPC through its receptor G_2A generated from hydrolysis or base-exchange reaction of LPE may be possible.

A relationship between the degree of MAPK phosphorylation and the concentration of LPE, S1P, or LPS was preliminarily examined. The minimal concentrations of LPE necessary for the induction of MAPK phosphorylation were equal or rather lower than those of S1P or LPC (data not shown). Therefore, it is difficult to assume that LPE is converted into S1P, LPC, or LPA and induces the phosphorylation of MAPK. Although the possibility that LPE induces the phosphorylation of MAPK through EGFR was suggested by this study, it could not be excluded that LPE directly stimulates G_2A and/or $S1P_1$, the receptors of S1P, LPC, or LPA. Kinetic analysis of LPE binding to these receptors is an urgent matter to clarify the mechanisms of LPE signal transduction.

As shown in Fig. 2, the major fatty acids bound to GLPE were oleic and linoleic acids. The contribution of a fatty acid base to pharmacological activity might be small, because the activities of synthetic 1-myristoyl-LPE, 1-palmitoyl-LPE, and 1-oleoyl-LPE were almost equal to those of GLPE. Moreover, it is clear that LPE itself induces the activation of ERK, because the GLPE activity was equal to the synthetic LPE activity.

In this study, the effects of LPE on the MAPK cascade have been partly clarified. The MAPK signal cascade is known to regulate cell growth and differentiation (44–46). For instance, tyrosine kinase receptors on cell membranes are activated by growth factors, and their signals are transduced to Raf, MEK1/2, ERK1/2, Elk1, and/or p90RSK. Because activation of ERK1/2 by GLPE was suppressed by a MEK1/2 inhibitor, we may assume that GLPE activated ERK1/2 via MEK1/2. We also tested the effect of a Raf-1 kinase inhibitor (5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]2-indolinone) (47) on the activation of ERK1/2 by GLPE. After PC12 cells were treated with Raf-1 kinase inhibitor, NGF, an activator of Raf-1/MEK/ERK, was added to the medium. ERK1/2 of PC12 cells was phosphorylated by the addition of NGF as well as in the Raf-1 kinase inhibitor-free medium. Thus, it has been confirmed that the Raf-1 kinase inhibitor mentioned above does not inhibit the activity of Raf in PC12 cells. To clarify the effect of GLPE on Raf, it will be necessary to use another Raf-1 kinase inhibitor that has better cell membrane permeability or to try another method, such as Raf-1 kinase assay (48).

The results shown in Fig. 5 suggest that EGFR, not the NGF receptor or pertussis toxin-sensitive GPCR, was necessary for the activation of ERK1/2 by GLPE. It is known from a recent study (23) that LPA, an analog of LPE, activates both EGFRs and pertussis toxin-sensitive GPCRs of PC12 cells, resulting in the induction of activation of the MAPK pathway. On the other hand, because PTX was irrelevant to the activation of ERK1/2 by GLPE, LPE would appear to differ from LPA in terms of pathway, as it induces the activation of ERK1/2 chiefly via the EGFR. It is known that U0126 binds to MEK1/2 of all types and inhibits the phosphorylation of ERK1/2, whereas PD98059 only binds to MEK of inactive type and inhibits the phosphorylation of MEK by Raf (49). The possibility that the phosphorylation of MEK by Raf is unnecessary to the phosphorylation of ERK by GLPE is likely, because the phosphorylation of ERK1/2 induced by GLPE was inhibited weakly by PD98059 but strongly by U0126. Elucidation of the signal pathway from the EGFR to MEK1/2 is an important issue to be resolved in the future.

From earlier experimental results showing a relationship between the fatty acid species bound to LPA and biological activity, 1-oleoyl-LPA had the strongest activity (50). However, the fatty acid species associated with LPE in our study did not appear to be related to the activity of LPE. We assume that the difference of the interactive functional group between LPE and LPA was responsible for the difference in the effects of the bound fatty acid on activity.

In conclusion, we found that GLPE induced the activation of the MAPK cascade via EGFR-MEK1/2-ERK1/2 in PC12 cells, which resulted in neuronal differentiation and suppression of serum deprivation-induced apoptosis. Our results clarified for the first time the involvement of the MAPK signal cascade in LPE actions, such as the induction of neuronal differentiation and the suppression of apoptosis. Thus, the clinical application of LPE, a low-molecular-weight compound having neuroprotective and neurotrophic activities, is hopeful. The potential use of LPE to prevent and treat neurological disorders such as Alzheimer's disease and the neuronal apoptosis that occurs after brain ischemia should be evaluated in the future.

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