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Lysophosphatidylcholine enhances NGF-induced MAPK and Akt signals through the extracellular domain of TrkA in PC12 cells☆

Wuhanqimuge[a], Asako Itakura[a], Yuri Matsuki[b], Masato Tanaka[b], Manabu Arioka[a,*]

[a] Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Department of Biotechnology, Tokyo Denki University, Ishizaka, Hatoyama-machi, Hiki-gun, Saitama 350-0394, Japan

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ABSTRACT

Lysophosphatidylcholine (LPC) is one of the major lysophospholipids mainly generated by phospholipase A_2 (PLA_2)-mediated hydrolysis of phosphatidylcholine (PC). We previously found that LPC displays neurotrophin-like activity in the rat pheochromocytoma PC12 cells and in cerebellar granule neurons, but the molecular mechanism remains unclear. We report here that LPC specifically enhances nerve growth factor (NGF)-induced signals in PC12 cells. When PC12 cells were treated with NGF, MAPK was phosphorylated, but this phosphorylation was significantly elevated when LPC was added together. In accordance, NGF-induced expression of immediate early genes, c-*fos* and *NGF-IA*, was upregulated by LPC. Phosphorylation of the upstream components, MEK and NGF receptor TrkA, was also promoted by LPC, which was in line with increased phosphorylation of Akt. In contrast, LPC did not enhance epidermal growth factor (EGF)-, basic fibroblast growth factor-, or insulin-like growth factor-1-induced signals. Studies using TrkA/EGF receptor chimeras demonstrated that the extracellular domain, but not the transmembrane or intracellular domains, of TrkA is responsible for the effect of LPC. Exogenously-added secretory PLA_2 ($sPLA_2$) enhanced NGF-induced MAPK phosphorylation at a comparable level to LPC, suggesting that LPC generated *in situ* by $sPLA_2$-mediated hydrolysis of membrane PC stimulated NGF-TrkA signal. Taken together, these results indicate a specific role and function of LPC on NGF-TrkA signaling pathway.

1. Introduction

Neurotrophins, including nerve growth factor (NGF) [1], brain-derived neurotrophic factor (BDNF) [2], neurotrophin-3 (NT-3) [3], and neurotrophin-4 [4], are a family of small secreted proteins. They have similar structure and functions, and play essential roles in maintaining the physiological activity of neurons through regulating their survival, growth, and differentiation, as well as the synaptic formation, plasticity, and other associated actions of neurons throughout the developmental processes [5–10]. NGF, the most extensively-characterized neurotrophin, is known to be generated from nonneuronal cells of target tissues, such as skin, muscle, testis, and salivary glands [5,6]. NGF acts through two classes of receptors: tropomyosin-related kinase A (TrkA), carrying an intrinsic tyrosine kinase activity in its intracellular domain, and the receptor p75 for neurotrophins ($p75^{NTR}$) that belongs to the death receptor family [6,7]. Binding of NGF to TrkA induces the auto-phosphorylation of TrkA at tyrosine residues and its kinase activity followed by the activation of Ras-mitogen-activated protein kinase (MAPK) cascade [6,7,11,12]. Activated TrkA also transmits signals to the phosphatidylinositol 3-kinase (PI3K)-Akt and phospholipase C-γ pathways [6,7,12]. These pathways finally lead to the expression of immediate early genes, such as c-*fos* and *NGF-IA*, which are involved in cell proliferation, differentiation, and survival [13].

Previously, we found that treatment of PC12 cells with secretory phospholipase A_2 ($sPLA_2$) induces phosphorylation/activation of MAPK, neuritogenesis, and differentiation into a sympathetic neuron-like phenotype, as observed in NGF-treated PC12 cells [14,15]. We also found that $sPLA_2$ protects cerebellar granule neurons (CGNs)

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Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CGNs, cerebellar granule neurons; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; EGFR, EGF receptor; GPCR, G protein-coupled receptors; IGF-1, insulin-like growth factor-1; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PC, phosphatidylcholine; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; $sPLA_2$, secretory PLA_2; TTBS, Tris-buffered saline containing 0.01% Tween 20.

* Corresponding author. Tel.: +81 3 5841 8230; fax: +81 3 5841 8033.
E-mail address: arioka@mail.ecc.u-tokyo.ac.jp (M. Arioka).

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from apoptosis, mimicking the action of BDNF [16]. Phospholipase A_2 (PLA_2) is comprised of a diverse class of enzymes which catalyze the hydrolysis of the *sn*-2 ester bond of phospholipids to liberate free fatty acids and lysophospholipids. They are mainly classified into four groups: $sPLA_2$, cytosolic PLA_2, calcium-independent PLA_2, and platelet-activating factor acetylhydrolase family [17]. It is widely recognized that through releasing lysophospholipids and fatty acids, especially arachidonic acid, PLA_2s play important roles in numerous cellular processes [18,19]. Interestingly, among the $sPLA_2$ isozymes tested, $sPLA_2$-X, but not $sPLA_2$-IB nor $sPLA_2$-IIA, induced neurite outgrowth in PC12 cells [15] and protected CGNs from apoptosis [16]. Subsequent studies have shown that the neurotrophin-like activity of $sPLA_2$ is associated with the release of lysophosphatidylcholine (LPC). Indeed, LPC added to PC12 and CGNs cultures recapitulated the neurotrophin-like activities of $sPLA_2$ [20,21].

LPC is known as a bioactive lipid mostly released from the plasma membrane and lipoprotein through the hydrolytic degradation of phosphatidylcholine (PC) by PLA_2. LPC can also be generated by endothelial lipase or by lecithin-cholesterol acyltransferase [22,23]. The level of LPC is increased in oxidized low density lipoprotein (Ox-LDL) and it is likely to play a critical role in the atherogenic effect of Ox-LDL [24]. LPC has been shown to be involved in various cellular responses, such as increasing intracellular Ca^{2+}, proinflammatory cytokines, and the expression of heparin-binding epidermal growth factor (EGF)-like growth factor gene. It was also reported that LPC induces the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, attracts phagocytes to apoptotic cells, and induces chemotaxis in other types of cells [24–26]. However, the mechanisms are still poorly understood. Our previous findings and these open questions prompted us to further investigate the effect of LPC on NGF signals in PC12 cells.

In this study, we found that LPC specifically enhances NGF-TrkA signals in PC12 cells. Signals from other growth factors such as EGF were not affected by LPC. Using TrkA/EGF receptor chimeras, we identified the extracellular domain of TrkA as the critical region for the effect of LPC. In addition, exogenously-added $sPLA_2$ also caused a similar enhancement of NGF-induced signal. These findings implicate a specific and functional interaction between LPC and NGF-TrkA system.

2. Results

2.1. LPC enhances NGF-induced MAPK phosphorylation in PC12 cells

In our previous study, we found that the release of LPC is involved in $sPLA_2$-induced neuronal differentiation of PC12 cells [20]. Since NGF and LPC activate similar but distinct signaling pathways (i.e. NGF acts through its receptor TrkA, while $sPLA_2$ activates L-type Ca^{2+} channel), we asked whether signaling cross-talk between NGF and LPC occurs in PC12 cells. To this end, cells were treated with NGF or LPC alone, or NGF plus LPC at specified concentrations for 10 min; NGF-induced MAPK phosphorylation in PC12 cells is commonly observed at this time point. Then, phosphorylation of MAPK (Erk1/2) was analyzed by Western blotting. PC12 cells express two similar MAPKs called Erk1 (44 kDa) and Erk2 (42 kDa) which are phosphorylated on specific threonine and tyrosine residues upon NGF treatment. As shown in Fig. 1, treatment of cells with NGF (10 and 50 ng/ml) triggered MAPK phosphorylation, whereas treatment with LPC alone (C16:0; 0.1 and 1 μM) failed to induce it. Interestingly, significant increase in MAPK phosphorylation was observed when cells were treated with NGF together with LPC; at the highest, LPC (1 μM) enhanced NGF (50 ng/ml)-induced phosphorylation of MAPK by three- to four-fold compared to the control (NGF alone, Fig. 1A and B), and this increase was dose-dependent of LPC used. At lower NGF concentration (10 ng/ml), the effect of LPC was smaller, but LPC consistently increased NGF-induced MAPK phosphorylation as shown in Fig. 1A.

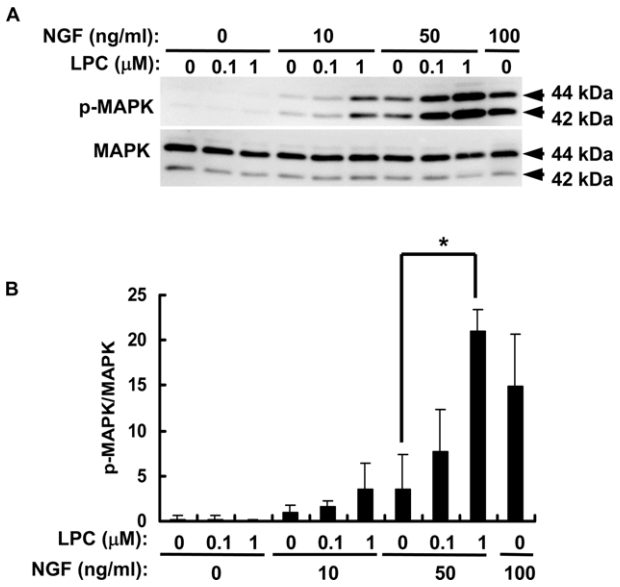

Fig. 1. LPC enhances NGF-induced MAPK phosphorylation in PC12 cells. PC12 cells were treated with vehicle control (DMEM plus methanol), LPC (0.1 and 1 μM) or NGF (10, 50, and 100 ng/ml) alone, or NGF and LPC together as indicated for 10 min. Cell lysis was collected and subjected to Western blotting as described in Section 4. (A) Phosphorylated MAPK (p-MAPK) and total MAPK (MAPK) were analyzed by Western blotting using phospho-p44/42 (Thr202/Tyr204) MAPK and p44/42 MAPK primary antibodies, respectively. (B) The amounts of p-MAPK and MAPK were quantified and the relative ratio of p-MAPK vs MAPK in each condition was calculated. Data are means ± SD of three independent experiments. $^*p < 0.01$ by one-way ANOVA.

This result implicates that a cross-talk between NGF and LPC signaling pathways exists in PC12 cells.

2.2 LPC containing fatty acyl chain longer than C14, but not other lysophospholipids tested, display significant enhancement of NGF-induced MAPK phosphorylation

The result shown in Fig. 1 prompted us to examine whether other lysophospholipids bearing different headgroups, i.e. lysophosphatidic acid (LPA), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS) display similar enhancement of NGF-induced MAPK phosphorylation. The result shows that these lysophospholipids also enhanced NGF-induced MAPK phosphorylation, but at significantly lower level than LPC (Fig. 2A and B), implying a special role of LPC on NGF-induced signaling in PC12 cells.

It has been reported that LPC species carrying different length of acyl chain and the degree of unsaturation play different biological roles [27–29]. Therefore, the effect of LPC species, C12:0, C14:0, C16:0, C18:0, and C18:1, on NGF-induced MAPK phosphorylation was examined. As shown in Fig. 2C and D, LPC carrying fatty acyl chains C14:0, C16:0, C18:0 and C18:1 was effective in the promotion of NGF-induced MAPK phosphorylation to a similar extent, whereas LPC C12:0 was ineffective. LPC C16:0 was used in the following experiments, since C16:0 is the most abundant molecular species of LPC.

2.3. LPC upregulates the expression of NGF-induced immediate early genes, c-fos and NGF-IA

c-*fos* and *NGF-IA* are two major immediate early genes which are rapidly transcribed in response to many extracellular stimuli, including NGF, and are early components of a series of transcriptional events necessary for initiation and maintenance of differentiation. The maximal increase in the expression of c-*fos* and *NGF-1A* by NGF is observed

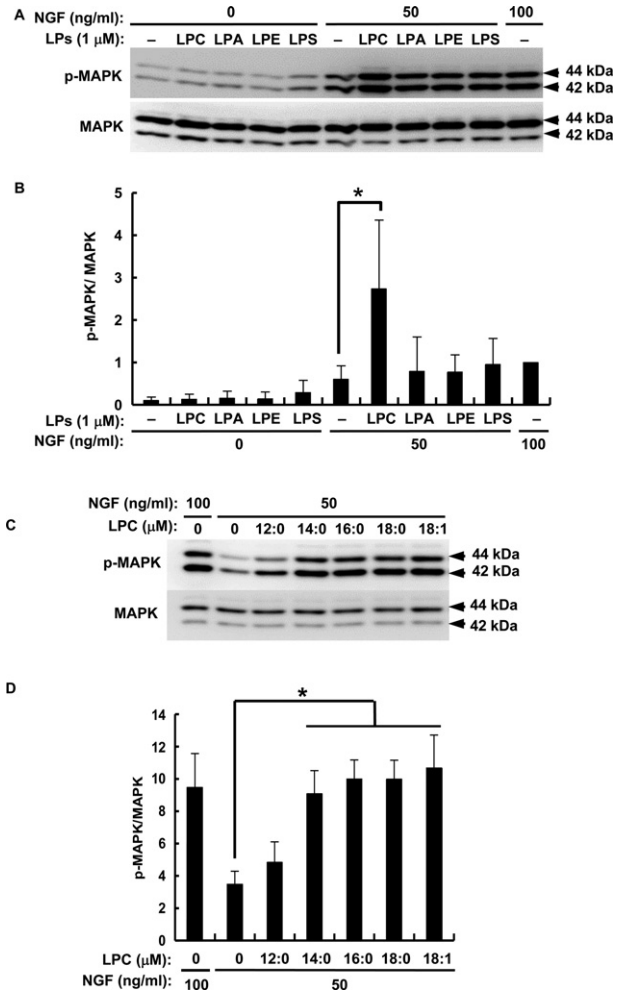

Fig. 2. LPC containing fatty acyl chain longer than C14, but not other tested lysophospholipids, show significant enhancement of NGF-induced MAPK phosphorylation in PC12 cells. (A and B) PC12 cells were treated with vehicle control (DMEM plus methanol), LPs (LPC, LPA, LPE, or LPS at 1 μM) or NGF (50 and 100 ng/ml) alone, or NGF and LPs together for 10 min. (C and D) PC12 cells were treated with NGF (50 and 100 ng/ml) alone or NGF (50 ng/ml) together with LPC (1 μM) containing fatty acyl chains of various length for 10 min. (A and C) show p-MAPK and total MAPK analysis by Western blotting. In (B and D), the amounts of p-MAPK and total MAPK were quantified and the relative ratio of p-MAPK vs MAPK in each condition was calculated. Data are means ± SD of four (B) or three (D) independent experiments. $^*p < 0.05$ by one-way ANOVA.

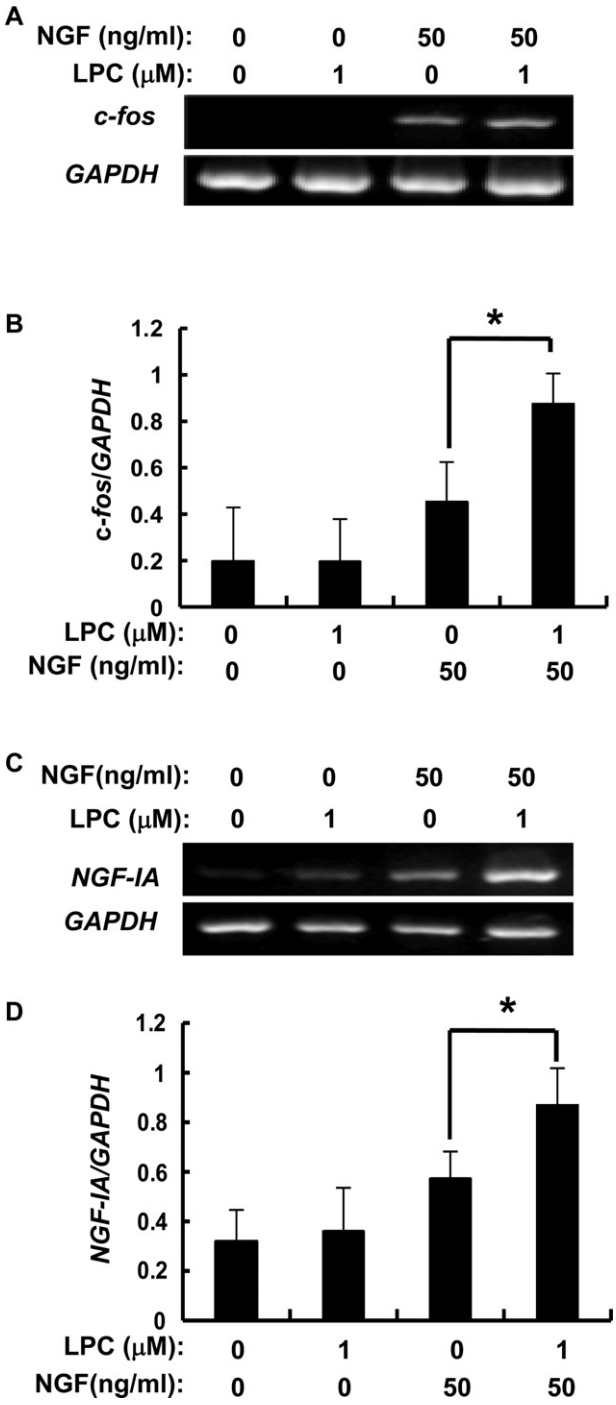

Fig. 3. LPC upregulates the expression of NGF-induced immediate early genes, c-fos and NGF-IA in PC12 cells. PC12 cells were treated with vehicle control (DMEM plus methanol), LPC (1 μM) or NGF (50 and 100 ng/ml) alone, or NGF (50 ng/ml) together with LPC (1 μM) for 30 min. Total RNA was isolated and reverse transcribed using the random primer. (A and C) The expression levels of c-*fos*, *NGF-IA*, and *GAPDH* were analyzed by RT-PCR. (B and D) The expression levels of c-*fos*, *NGF-IA*, and *GAPDH* were measured by quantitative real-time PCR. The amounts of transcripts for c-*fos* or *NGF-IA* relative to *GAPDH* were calculated by setting the value for NGF (100 ng/ml; not shown) at 1. Data are means ± SD of three independent experiments. $^*p < 0.05$ by one-way ANOVA.

at 30 min [13]. To examine whether LPC affects the expression of c-*fos* and *NGF-IA* upon NGF treatment in PC12 cells, cells were stimulated with NGF in the presence or absence of LPC for 30 min, and the expression of both genes was measured by semi-quantitative and quantitative real time PCR. Consistent with the result shown in Fig. 1, NGF induced the expression of c-*fos* and *NGF-IA*, and the addition of LPC significantly upregulated the expression of both genes, while LPC alone failed to induce the expression of both genes (Fig. 3A–D). This result shows that enhanced MAPK phosphorylation by LPC results in the elevation of c-*fos* and *NGF-IA* expression at the transcriptional level, suggesting again a functional role of LPC on NGF-induced signaling pathway.

2.4. LPC promotes NGF-induced MAPK phosphorylation through enhancing the phosphorylation of MEK and the receptor TrkA

To pinpoint the cellular component at which the NGF signal is augmented by LPC, we examined MEK phosphorylation, which is just upstream of MAPK activation. We observed that phosphorylation of MEK1/2 triggered by NGF was significantly enhanced by LPC (Fig. 4A and B), indicating that LPC acts on MEK or at the upstream of MEK. LPC alone did not induce MEK phosphorylation in PC12 cells (Fig. S1).

We next tested whether the enhancement of NGF-induced MAPK and MEK phosphorylation by LPC occurs via augmentation of the

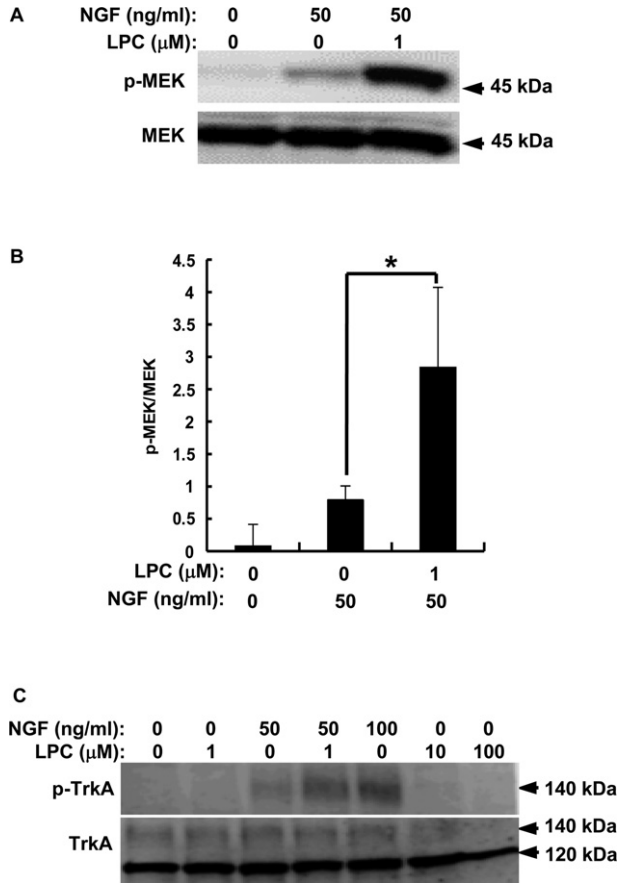

Fig. 4. LPC enhances NGF-induced MAPK phosphorylation through enhancing the phosphorylation of MEK and receptor TrkA. (A) PC12 cells were treated with vehicle control (DMEM plus methanol), or NGF (50 ng/ml) in the absence or presence of LPC (1 μM) for 5 min. Phosphorylated MEK (p-MEK) and total MEK (MEK) were analyzed using anti-phospho-MEK1/2 (Ser217/221) and anti-MEK1/2 antibodies, respectively. (B) The amounts of p-MEK and total MEK were quantified and the relative ratio of p-MEK vs total MEK was calculated. Data are means ± SD of three independent experiments. $^*p < 0.05$ by one-way ANOVA. (C) PC12 cells were treated with vehicle control (DMEM plus methanol), NGF (50 and 100 ng/ml) or LPC (1, 10, and 100 μM) alone, or NGF (50 ng/ml) together with LPC (1 μM) as indicated, for 5 min. Phosphorylated TrkA (p-TrkA) and total TrkA (TrkA) were analyzed using anti-phospho-TrkA (Tyr490) and anti-TrkA primary antibodies, respectively. The images shown are representative of three independent experiments which essentially gave similar results.

activation of NGF receptor, TrkA. NGF (50 ng/ml)-induced phosphorylation of TrkA at tyrosine 490, which is known to be the important site for the induction of MAPK and Akt signaling cascades [7], was significantly enhanced by the addition of LPC (1 μM) (Fig. 4C). LPC alone (1, 10, or 100 μM) did not induce the phosphorylation of TrkA. Taken together, results obtained in Figs. 1–4 indicate that LPC upregulates NGF-induced signaling by enhancing NGF-induced activation of TrkA.

2.5. Akt phosphorylation induced by NGF, but not by IGF-1, was enhanced by LPC

As mentioned above, NGF also induces the activation of PI3K-Akt signaling cascade at the downstream of TrkA. We then asked that if LPC also affects NGF-induced Akt phosphorylation in PC12 cells. To test this, cells stimulated with NGF in the presence or absence of LPC were analyzed for Akt phosphorylation using the antibodies against phospho-Akt (Ser473) and Akt. As shown in Fig. 5A and B, NGF-induced Akt phosphorylation was significantly increased by LPC. No significant difference was detected in Akt protein levels.

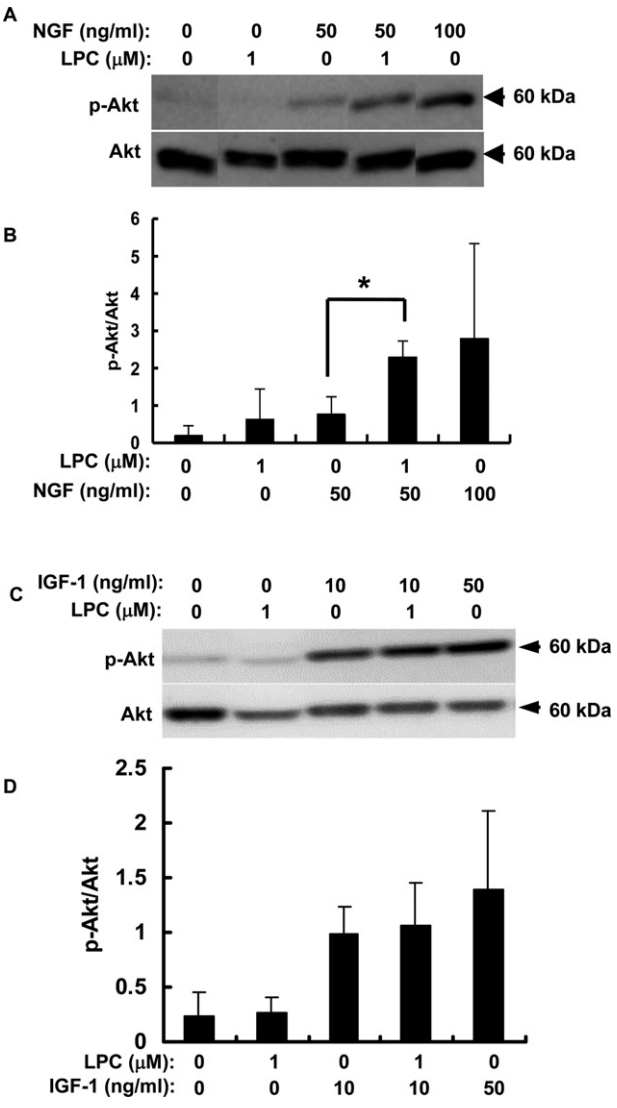

Fig. 5. LPC also enhances NGF-induced, but not IGF-1-induced, Akt phosphorylation. (A and B) PC12 cells were treated with vehicle control (DMEM plus methanol), LPC (1 μM) or NGF (50 and 100 ng/ml) alone, or NGF (50 ng/ml) together with LPC (1 μM) for 10 min. (C and D), PC12 cells were treated with vehicle control (DMEM plus methanol), LPC (1 μM) or IGF-1 (10 and 50 ng/ml) alone, or IGF-1 (10 ng/ml) together with LPC (1 μM) for 30 min. (A and C) Phosphorylated Akt (p-Akt) and total Akt (Akt) were analyzed by Western blotting using anti-phospho-Akt (Ser473) and anti-Akt primary antibodies, respectively. (B and D) The amounts of p-Akt and total Akt were quantified and the relative ratio of p-Akt vs total Akt was calculated. Data are means ± SD of at least three independent experiments. $^*p < 0.05$ one-way ANOVA.

Insulin-like growth factor-1 (IGF-1) is a polypeptide trophic factor playing important roles in the survival and differentiation of both neuronal and non-neuronal cells. It has been shown that IGF-1 induces Akt survival pathway in PC12 cells [30]. Since LPC enhanced NGF-induced Akt phosphorylation, we examined if LPC also augments IGF-1-induced Akt phosphorylation. However, no significant enhancement of Akt phosphorylation was observed by LPC (Fig. 5C and D). Thus, LPC does not affect IGF-1-induced Akt signaling, implying that the effect of LPC is specific to NGF-TrkA.

2.6. LPC does not affect EGF- or FGF-induced MAPK phosphorylation and EGF-induced EGF receptor phosphorylation

To test whether LPC also promotes MAPK phosphorylation triggered by other growth factors, the effect of LPC on the signals elicited by various growth factors, EGF and basic fibroblast growth factor

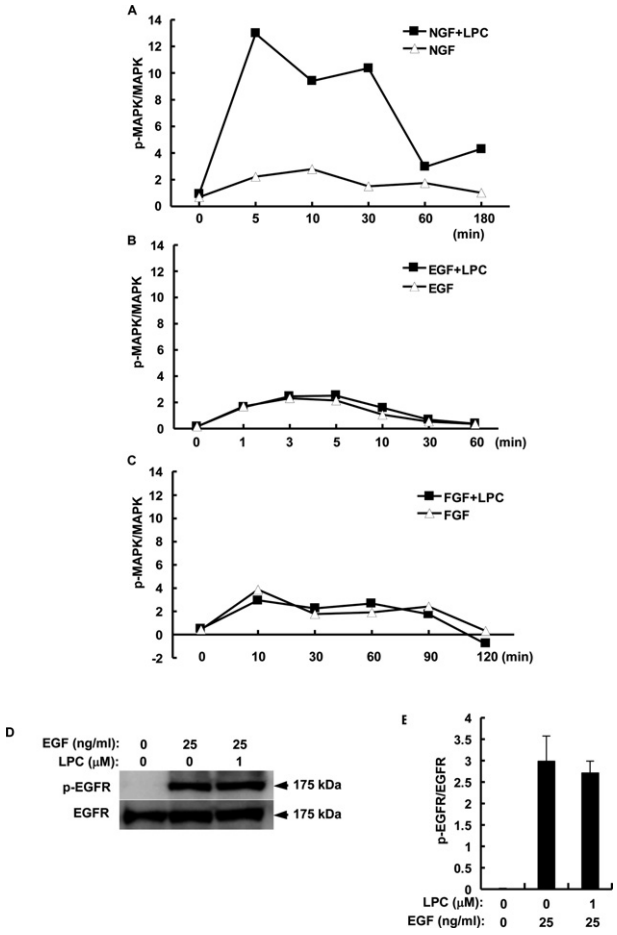

Fig. 6. LPC does not enhance EGF- or FGF-induced MAPK phosphorylation, and EGF-induced EGF receptor phosphorylation. (A–C) PC12 cells were stimulated with NGF (50 ng/m) in (A), EGF (25 ng/ml) in (B), or FGF (50 ng/ml) in (C), alone or together with LPC (1 μM) in the time-course experiments as indicated. Then, p-MAPK and total MAPK were analyzed by Western blotting. The graphs shown are a representative of at least three independent experiments which essentially gave similar results. (D and E) PC12 cells were treated with vehicle control (DMEM plus methanol), or EGF (25 ng/ml) in the absence or presence of LPC (1 μM) for 2 min. Phosphorylated EGF receptor (p-EGFR) and total EGFR (EGFR) were analyzed by Western blotting in (D) using anti-phospho-EGFR (Tyr1173) and anti-EGFR primary antibodies, respectively. The amounts of p-EGFR and total EGFR were quantified and the relative ratio of p-EGFR vs total EGFR was calculated in (E). Data are means ± SD of two independent experiments.

(bFGF), as well as NGF, was analyzed in the time-course experiments. As shown in Fig. 6A, LPC significantly enhanced NGF-induced MAPK phosphorylation, and the sustained effect was observed for as long as 30 min. In addition, LPC accelerated the cellular response as the peak of NGF-induced MAPK phosphorylation moved from 10 to 5 min (Fig. 6A). In contrast, no enhanced phosphorylation of MAPK by LPC was detected when cells were treated with EGF (Fig. 6B) or bFGF (Fig. 6C). These data pose again an interesting possibility that LPC acts specifically on NGF-TrkA signaling pathway.

To understand why LPC failed to enhance EGF-induced MAPK phosphorylation, the effect of LPC on EGF-induced EGF receptor (EGFR) phosphorylation was tested. In line with the result showing that EGF-elicited MAPK phosphorylation was not enhanced by LPC, EGF-induced autophosphorylation of EGFR at Tyr1173, which is involved in MAPK activation, was not affected by LPC (Fig. 6D and E). Considering that EGF at 25 ng/ml might maximally induced EGFR phosphorylation, so that no further increase occurred by LPC, the effect of LPC on EGFR phosphorylation induced by EGF at 5 and 10 ng/ml was also examined; LPC did not affect EGF-induced EGFR phosphorylation (data not shown). Taken together, the results obtained clearly show that LPC specifically promotes NGF-induced MAPK and Akt phosphorylation through enhancing the activation of TrkA.

2.7. Extracellular domain of TrkA is responsible for the effect of LPC on NGF-dependent MAPK phosphorylation

Results in Figs. 4C and 6D showing that TrkA, but not EGFR, was responsive to the effect of LPC suggest that LPC plays a specific role on the activation of TrkA, rather than influencing the signals evoked by growth factor-receptor tyrosine kinase in general. To further understand the mechanism underlying the action of LPC, we next aimed to determine the domain(s) of TrkA involved in the effect of LPC on NGF-induced MAPK phosphorylation. To this end, we constructed TrkA/EGFR chimeras, C1, C2, C3, and C4, by swapping the extracellular (EC), transmembrane (TM), and intracellular (IC) domains between TrkA and EGFR (Fig. 7A). We firstly confirmed that untransfected and vector-transfected CHO-K1 cells do not respond to either NGF or EGF (i.e. MAPK was not phosphorylated upon NGF or EGF treatments; Fig. 7B, upper). Next, we transfected TrkA, EGFR, and TrkA/EGFR chimeras to CHO-K1 cells, respectively, and tested MAPK phosphorylation upon various stimuli. Expression of these receptors was confirmed by Western blotting using anti-GFP-antibody (Fig. 7B, bottom). In TrkA-transfected cells, addition of NGF weakly induced MAPK phosphorylation, and when LPC was added together, it was significantly increased (Fig. 7C and D). In EGFR-transfected cells, EGF induced MAPK phosphorylation, but this was not affected by LPC (Fig. 7E and F). These are consistent with the results we obtained in PC12 cells. In C1 (TrkA EC/EGFR TM + IC chimera)-transfected cells, NGF induced MAPK phosphorylation, which was further enhanced by LPC, as was seen in the TrkA-transfected cells (Fig. 7G and H). Similar result was obtained in the C3 (TrkA EC + TM/EGFR IC chimera)-transfected cells (Fig. 7K and L). These results show that the extracellular domain of TrkA responds to LPC in enhancing NGF-induced MAPK phosphorylation. Conversely, C2 (EGFR EC/TrkA TM + IC chimera; Fig. 7I and J) or C4 (EGFR EC + TM/TrkA IC chimera)-transfected cells (Fig. 7M and N) responded to EGF i.e. MAPK was strongly phosphorylated upon EGF treatment, since these cells express the extracellular domain of EGFR. However, no significant enhancement of MAPK phosphorylation was observed by LPC, although these cells express transmembrane or transmembrane plus intracellular domains of TrkA. Taken together, these results indicate that the extracellular domain, but not the transmembrane and intracellular domains, of TrkA is necessary and sufficient for mediating the effect of LPC on NGF-induced MAPK phosphorylation.

2.8. $sPLA_2$ enhances NGF-induced MAPK phosphorylation at a comparable level to LPC

We previously demonstrated that $sPLA_2$ displays neurotrophin-like activities, such as neurite-induction in PC12 cells and rescue of CGNs from apoptosis. These effects of $sPLA_2$ were essentially attributable to the generation of LPC [20,21]. Here we tested if exogenously-added $sPLA_2$ also enhances NGF-induced MAPK phosphorylation. As shown in Fig. 8, NGF-induced MAPK phosphorylation was greatly elevated by $sPLA_2$, and the degree of enhancement was similar to that by LPC, implying that LPC generated locally by $sPLA_2$-mediated hydrolysis of PC in the plasma membrane activates NGF-TrkA signaling pathway *in situ*.

3. Discussion

In this study, we have demonstrated that LPC specifically promotes NGF-induced MAPK and Akt signaling pathways in PC12 cells. Signals elicited by other growth factors, EGF, bFGF, and IGF-1, were not elevated by LPC, indicating that the effect of LPC is specific to NGF-TrkA system. To identify the cellular component(s) at which LPC

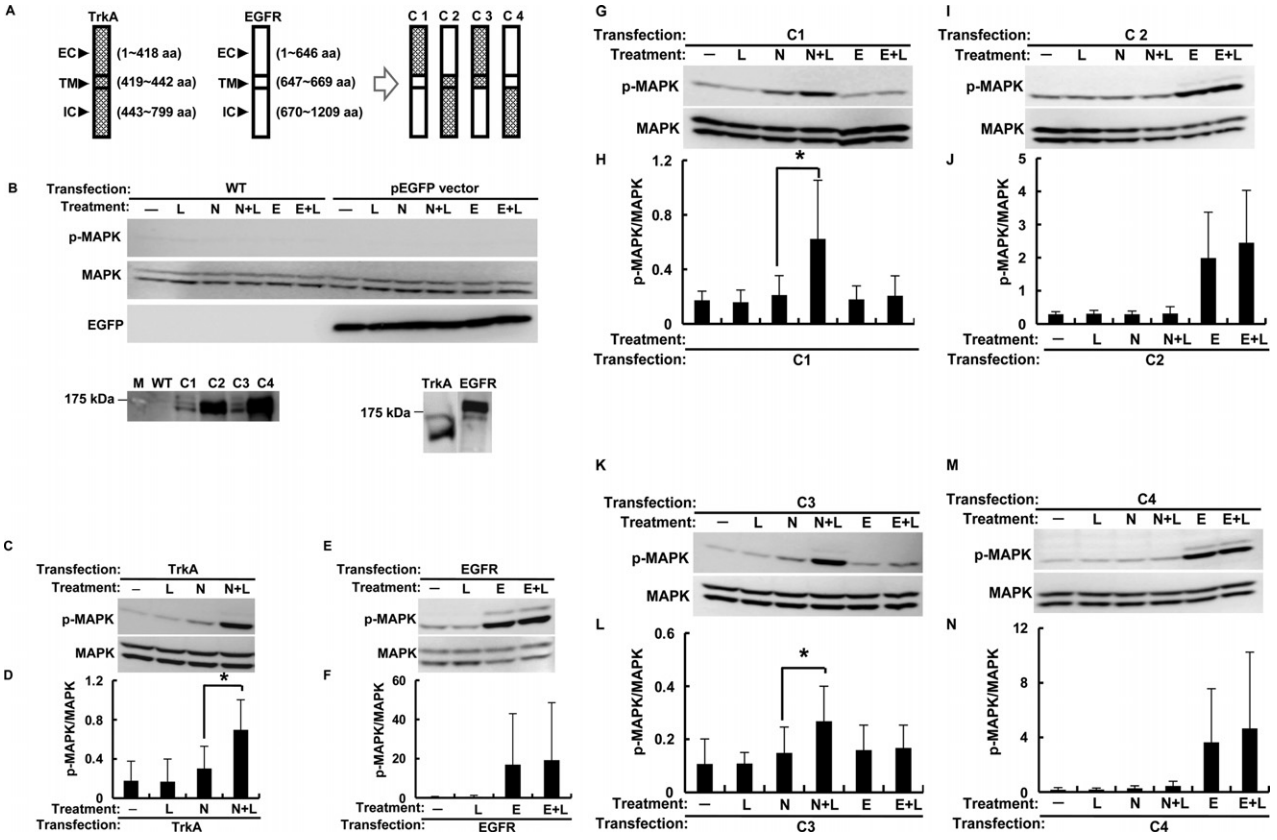

Fig. 7. LPC enhances NGF-dependent MAPK and Akt signals through the extracellular domain of TrkA. (A) Schematic representation of TrkA, EGFR, and TrkA/EGFR chimeric receptors C1, C2, C3, and C4. (B) CHO-K1 cells were untransfected (WT), or transfected with pEGFP vector, C1-pEGFP (C1), C2-pEGFP (C2), C3-pEGFP (C3), C4-pEGFP (C4), TrkA-pEGFP (TrkA), or EGFR-pEGFP (EGFR) for 18–24 h. Then, p-MAPK and MAPK were analyzed by Western blotting after various treatments (upper). Expression of EGFP, C1, C2, C3, C4, TrkA, and EGFR was also detected using anti-GFP-antibody (bottom). (C–N) CHO-K1 cells were transiently transfected with TrkA (TrkA-pEGFP) in (C and D), EGFR (EGFR-pEGFP) in (E and F), C1 (C1-pEGFP) in (G and H), C2 (C2-pEGFP) in (I and J), C3 (C3-pEGFP) in (K and L), or C4 (C4-pEGFP) in (M and N), respectively. Cells were serum-starved for 1.5 h. (B–N) Cells were treated with vehicle control (DMEM plus methanol), LPC (L, 1 μM) or NGF (N, 50 ng/ml) alone, or NGF (50 ng/ml) together with LPC (1 μM, N + L), EGF (E, 25 ng/ml) or EGF (25 ng/ml) together with LPC (1 μM, E + L) for 5 min. In the upper panels, p-MAPK and total MAPK were analyzed by Western blotting. In the bottom panels (graphs), the amounts of p-MAPK and total MAPK were quantified and the relative ratio of p-MAPK/total MAPK in each condition was calculated. Data are means ± SD of three independent experiments. $^{*}p < 0.05$ by one-way ANOVA.

acts to potentiate NGF-TrkA signal, we examined the phosphorylation of MEK and TrkA, and found that LPC enhances TrkA phosphorylation induced by NGF. Result from TrkA/EGFR chimera study showed that the extracellular domain, but not the transmembrane and intracellular domains, of TrkA is critical for the effect of LPC. Together, the results presented in this study propose a unique role of LPC in NGF-TrkA signaling pathway.

Lysophospholipids with different headgroups, LPA, LPE, and LPS enhanced NGF-induced MAPK phosphorylation, but the level was lower than LPC (Fig. 2A and B). Interestingly, similar headgroup specificity of these lysophospholipids was observed in our previous studies wherein neurite outgrowth in PC12 cells and the cell survival of CGNs were examined; only LPC, but not other lysophospholipids, induced neurites in PC12 and rescued CGNs from apoptosis [20,21]. Furthermore, LPC with fatty acyl chains of C14:0, C16:0, C18:0, and C18:1, but not C12:0, enhanced NGF-induced MAPK phosphorylation in this study, and similar LPC species were effective on neurite outgrowth in PC12 cells and the cell survival of CGNs in our previous studies [20,21]. These results suggest the existence of specific and acyl chain-dependent role(s) of LPC in neuronal systems.

We previously found that when PC12 cells were treated with $sPLA_2$, LPC was released into the culture medium due to its hydrolytic activity. Release of LPC was also detected in the medium of PC12 cells treated with the culture supernatant of COS1 cells expressing $sPLA_2$-X, but not $sPLA_2$-IB and $sPLA_2$-IIA [20]. Another study has shown that the amount of LPC in extracted phospholipids from PC12 cells that had been infected with the adenovirus containing $sPLA_2$-X, but not $sPLA_2$-IIA and V for 3 days, was greatly increased compared to that from the control cells; around 15–20% of total PC was found to be converted to LPC [34]. Furthermore, the expression of $sPLA_2$s including $sPLA_2$-X in the skin is known to be increased during inflammation caused by UV irradiation. Recombinant $sPLA_2$-X promoted the tyrosinase activity and dendricity in human melanocytes that play important roles in the protection of skin from UV damage, which was mainly dependent on the release LPC [35]. Thus it is conceivable that LPC is locally generated *in vivo* and associated with some of the biological actions of $sPLA_2$.

It remains an open question how LPC acts to potentiate NGF-Trk signals. Accumulating evidence suggests the involvement of G protein-coupled receptors (GPCRs) such as G2A and GPR4 in the biological actions of LPC, such as the increase in transforming growth factor-b1 expression, and the barrier dysfunction induced by LPC [37,38]. Also, in our previous study, G2A mediates the neuritogenic action of LPC in PC12 cells [20]. Hence, we tested if G2A and GPR4 modulate the effect of LPC on NGF-induced signals by overexpressing G2A (or GPR4) in PC12 cells, or by co-expressing G2A (or GPR4) with TrkA in CHO-K1 cells. In both cases, however, the ability of LPC to promote NGF-induced MAPK phosphorylation was not affected (data not shown). Nevertheless, possibilities that the effect of LPC was mediated by different GPCR(s), like GPR11, another GPCR involved in the effect of LPC [39], as well as adenosine 2A receptor [40], PAC1 receptors [41], and endocannabinoid receptors [42] which were reported

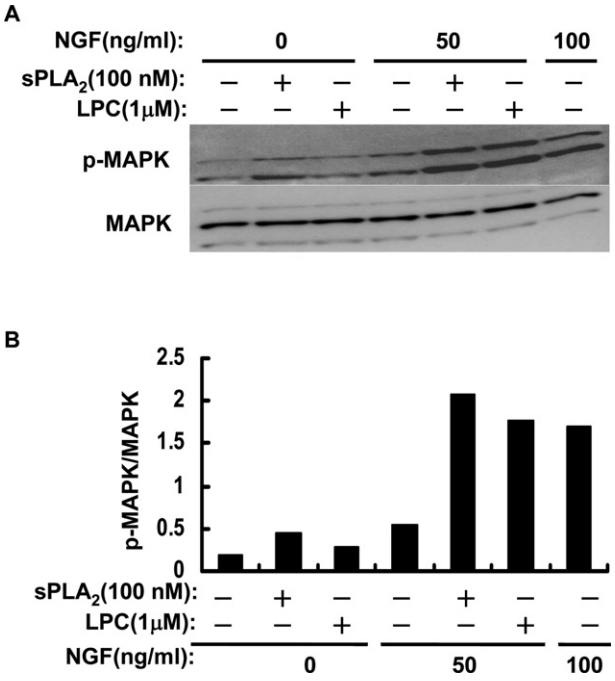

Fig. 8. $sPLA_2$ enhances NGF-induced MAPK phosphorylation at similar level to LPC. PC12 cells were stimulated with vehicle control (DMEM plus methanol), LPC (1 μM), $sPLA_2$ (100 nM), NGF (50 ng/ml), or NGF (50 ng/ml) together with LPC (1 μM) or $sPLA_2$ (100 nM) for 2 min. (A) p-MAPK and total MAPK were analyzed by Western blotting. (B) The amounts of p-MAPK and total MAPK were quantified and the relative ratio of p-MAPK vs total MAPK was calculated. One representative result from four independent experiments which gave similar results is shown.

to induce the activation of Trk receptors, need to be addressed.

Another possibility is that LPC might regulate the dimerization state of TrkA, since it is well accepted that NGF induces dimerization and autophosphorylation of TrkA, thereby activating the downstream signaling events. Recent studies have shown, however, that the majority of TrkA preforms dimers in the endoplasmic reticulum before reaching to the cell surface; NGF activates the preformed, yet inactive, TrkA dimer on the cell surface [11]. We examined the dimerization state of TrkA by performing a chemical cross-linking experiment, and found that LPC did not affect the formation of TrkA dimer in TrkA-transfected PC12 cells (data not shown). This result suggests that LPC does not act through enhancing/stabilizing the formation of TrkA dimers that occurs intracellularly, which is, in fact, compatible with our finding that LPC acts on the extracellular domain of TrkA.

The mechanism whereby LPC augments NGF-induced signals could be more direct. Studies using TrkA/EGFR chimeric receptors expressed in CHO-K1 cells showed that the extracellular domain of TrkA is responsible for the effect of LPC on NGF signals. The extracellular immunoglobulin-like subdomains of TrkA, D4 and D5, were reported to be important for NGF binding. When D4 and D5 were removed from TrkA, NGF binding was inhibited [43]. Subsequent studies showed that D5, located near the transmembrane region, is critical and sufficient for NGF binding [44,45]. LPC is an amphiphlic molecule and should distribute into both membrane and soluble compartments. Our data showing that the effect of LPC on the phosphorylation of TrkA was observed only in the presence of NGF, but not in its absence, might suggest that LPC evokes allosteric changes in the membrane-proximal D5 subdomain of TrkA, thereby modulating the affinity and/or stability of TrkA-NGF complex. Alternatively, LPC might affect TrkA activity by modulating the properties of lipid rafts where phosphorylated TrkA interact with the downstream effectors [46]. TrkA activity is also known to be regulated by GM1 ganglioside [47]. However, pretreatment of PC12 cells with methyl-β-cyclodextrin that disrupt lipid rafts failed to abolish the effect of LPC on NGF-induced MAPK phosphorylation (data not shown).

In contrast to other lysophospholipids including LPA, LPE, and LPS, the biological actions of which are mostly mediated by specific GPCRs and are observed at submicromolar or micromolar concentrations, most studies wherein the effects of LPC were examined used LPC at concentrations higher than 10 μM [31]. The concentration of LPC in the human plasma separated from fresh blood is around 190 μM, and it can be up to 800 μM in the blood plasma of other mammalian species. These facts have raised a critique on the existence of transmembrane signal transduction pathway(s) specific to LPC. However, the level of LPC in the cerebrospinal fluid (CSF) is only around 5 μM, since autotaxin abundantly expressed in the CSF converts LPC to LPA [27,32,33]. Furthermore, intravenously injected LPC (200 nM/kg) was found to protect neurons in the brain in an *in vivo* model of global ischemia in mice. In this model, 0.1% of intravenously injected LPC passed through the blood–brain barrier and entered the brain, 55% of which was in unmetabolized form. In addition, in an *in vitro* model of high glutamate-induced excitotoxicity of primarily cultured CGNs, LPC also significantly prevented the neuronal death [36]. These findings indicate that LPC is neuroprotective in the physiological conditions and might be a therapeutic candidate for preventing neuronal death, although the exact working concentration of LPC is unclear at present. Further studies are surely needed to elucidate the molecular mechanism by which LPC regulates the activation of TrkA, and whether or not the potentiation of NGF-TrkA signal is involved in the neuroprotective effect of LPC.

4. Experimental procedures

4.1. Materials

LPC used in this study is 1-palmitoyl-*sn*-glycero-3-phosphocholine (C16:0; Cat. No. 855675P). Other lysophospholipids used are: 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C12:0; 855475P), 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C14:0; 855575P), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C18:0; 855775P), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C:18:1; 845875P), lysophosphatidic acid (LPA; C16:0; 857123P), lysophosphatidylethanolamine (LPE; C16:0; 856705P), and lysophosphatidylserine (LPS; C18:1; 858143P). All these lysophospholipids were purchased from Avanti Polar Lipids. LPC, LPA, and LPS were dissolved in methanol. LPE was dissolved in DMSO. Nerve growth factor (NGF; NGF-301) and epidermal growth factor (EGF; EGF-201) were from Toyobo. Recombinant human insulin-like growth factor-1 (IGF-1; GPT-10011L) was obtained from Pepro Tech. Growth factors were dissolved in DMEM. $sPLA_2$ was prepared as described [15]. Vehicle control for various stimuli was prepared by mixing the same amount of DMEM and methanol.

Primary antibodies used are: phospho-p44/42 (Thr202/Tyr204) MAP kinase, #9101; p44/42 MAP kinase, #9102; phospho-Akt (Ser473), #9271; Akt, #9272; phospho-TrkA (Tyr490), #9141; TrkA #2505; phospho-EGF receptor (Tyr1173), #4407; EGF receptor, #2232; phospho-MEK1/2 (Ser217/221), #9121; and MEK1/2, #9122. All these antibodies were purchased from Cell Signaling Technology and used at 1:1000 dilution in Tris-buffered saline (TBS; 20 mM Tris–HCl (pH 7.5), 150 mM NaCl). Living colors® A.v. monoclonal antibody (JL-8), #632380 (Clontech), was used at 1:5000 dilution in TBS to detect EGFP fusion proteins. The secondary antibody, horseradish peroxidase-linked anti-rabbit-IgG (#7074; Cell Signaling Technology), was used at 1:2000 dilution in TBS containing 0.02% Tween 20 (TTBS). Peroxidase-labeled anti-mouse IgG (H + L), #PI-2000 (Vector), was used at 1:500 dilution in TBS.

4.2. Cell culture

Rat pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% fetal calf serum and 5% horse serum (DMEM (5/5)) at 37 °C in a humidified and CO_2-controlled (10%) incubator. Chinese hamster ovary K1 (CHO-K1) cells were maintained in DMEM supplemented with 10% fetal calf serum. Cells were kept with regular transfer of twice or more a week. PC12 cells were inoculated in collagen type 1 (rat tail)-coated 24-well culture plates at a density of 1×10^5 cells/well in Figs. 1, 2, 5, 6A–C, and 8, the same number of PC12 cells in 6-well plates in Figs. 4C, 6D, and 6E, or 60-mm dishes in Figs. 3, 4A and B, and allowed to grow until >80% confluence. Before cells were subjected to various treatments as specified in the text, cells were serum-starved for 1.5 h in DMEM in all cases. In Fig. 7, CHO-K1 cells were seeded at a density of 1×10^5 cells/well in 24-well culture plates, and allowed to grow until >80% confluence. 18–24 h after transfection, cells were serum-starved for 1.5 h in DMEM, and then subjected to various treatments as specified in the text.

4.3. Immunoblotting analysis

Cell lysis was collected in 1 × sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl (pH 6.8), 10% glycerol, 6% mercaptoethanol, 2% SDS, 0.5% bromophenol blue), 50 μl/well for 24-well plate, 100 μl/well for 6-well plate, and 300 μl/well for 60 mm dish, and boiled for 3 min. In each experiment, equal volume of cell lysate was subjected to electrophoresis on SDS gel containing 10% acrylamide. Proteins were transferred onto polyvinylidene fluoride (PVDF) microporous membrane (Millipore) for 45 min at 125 mA using a semi-dry blotter. The membrane was blocked with 5% skim milk for 1 h. Then, the membrane was incubated overnight at 4 °C with the primary antibodies as described. The membrane was washed with TTBS and incubated with the secondary antibody for 30 min or longer at room temperature with gentle shaking. Immunoreactive bands were visualized using the SuperSignal® WestPico Lumino/Enhancer (Pierce #1856136) and SuperSignal® WestPico Stable Peroxide (#1856135) solution, or Western Lightning® Ultra solutions from PerkinElmer, Inc. Imaging was then carried out using FUJI Image Reader. The amount of proteins was quantified by FUJI FILM Multi Gauge software.

4.4. Total RNA isolation and cDNA synthesis

Total RNA (10 μg) was extracted from PC12 cells after the various treatments as specified in Fig. 3, 5 μg of which was subjected to DNase treatment using RQ[1] RNase-Free DNase (Promega). Then, 0.6 μg of RNA was reverse transcribed to cDNA in a volume of 20 μl reaction using random primer and PrimeScript reverse transcriptase. After the reverse transcription reaction, 1 μl of cDNA from each case was proceeded to PCR reaction in a 25 μl of reaction mixture (95 °C for 5 min; 35 cycles of 94 °C for 30 s, 52.5 °C for 30 s (for *GAPDH*; 54.3 °C for 30 s for c-*fos*; 50 °C for 30 s for *NGF-IA*), and 72 °C for 30 s (for *GAPDH*; 72 °C for 40 s for c-*fos*; 72 °C for 1 min for *NGF-1A*); 72 °C for 5 min) using specific primer pairs: *GAPDH* (5′-GACCACAGTCCATGCCATCACT-3′ and 5′-TCCACCACCCTGTTGCTGTAG-3′), c-*fos* (5′-AGAATCCGAAGGGAAAGGAA-3′ and 5′-ATGATGCCGGAAACAAGAAG-3′), and *NGF-IA* (5′-CCACAACAACAGGGAGACCT-3′ and 5′-GGGATGGG TAGGAAGAGAGG-3′).

4.5. Semi-quantitative and quantitative RT-PCR

PCR products were subjected to electrophoresis on 2% agarose gel to confirm that each primer pair amplified a single product of predicted size, and to determine the relative expression level of c-*fos*, *NGF-IA*, and *GAPDH* in response to different treatments as described above. Also, transcript levels of c-*fos*, *NGF-IA*, and *GAPDH* were measured by quantitative real-time PCR using LightCycler® FastStart DNA Master SYBR Green I kit (Roche). The reaction was performed in a volume of 20 μl according to the manufacturer's instructions. In each reaction, 2 μl of cDNA was used. For each primer pair, PCR efficiency was determined by standard curve and the transcript levels of c-*fos* and *NGF-IA* were normalized against *GAPDH*. Quantitative real-time PCR (RT-PCR) experiments were independently performed three times and each experiment was done in triplicate.

4.6. Plasmids

To construct the plasmid TrkA-pEGFP for expression of TrkA fused with EGFP, a cDNA fragment encoding the full-length mouse TrkA, 799 amino acid-long, was amplified by PCR using oligonucleotides 5′-GGAATTCATGCTGCGAGGCCAGCGGCA-3′ and 5′-GGAATTCTGCCCAGAACGTCCAGGTAAC-3′. The resulting PCR product was digested with *Eco*R I, and was cloned into *Eco*R I site of pEGFP expression vector. To construct the plasmid EGFR-pEGFP for expression of EGFR fused with EGFP, a cDNA fragment encoding the full-length rat EGFR, 1209 amino acid-long, was amplified by PCR using oligonucleotides 5′-CTCGAGATGCGACCCTCAGGGACTGCGAGAACCAAGC-3′ and 5′-CTCGAGTGCTCCACTAAACTCACTG CTTGGCGGTGCCA-3′. This cDNA fragment was digested with *Xho* I, and subcloned into the expressing vector pEGFP at *Xho* I site (EGFR-pEGFP). TrkA/EGFR chimeric receptors were constructed by swapping each domain between TrkA and EGFR. cDNA fragments encoding the extracellular domain (EC), extracellular and transmembrane domains (EC-TM), transmembrane and intracellular domains (TM-IC), and intracellular domain (IC) of both TrkA and EGFR were amplified by PCR, using TrkA-pEGFP and EGFR-pEGFP as templates, respectively. Resultant fragments were fused by overlapping PCR strategy to create chimeric cDNAs. Chimeric receptor 1 (C1) is composed of EC of TrkA and TM-IC of EGFR. C2 contains EC of EGFR and TM-IC of TrkA. C3 contains EC-TM of TrkA and IC of EGFR. C4 contains EC-TM of EGFR and IC of TrkA. Chimeric cDNAs for C1, C2, C3, and C4 was digested with *Xho* I and introduced into pEGFP vector at *Xho* I site. Oligonucleotides used are: 5′-CTCGAGATGCT GCGAGGCCAGCGGCTCGG-3′, 5′-CCCACAATCCCAGTGGCGATAGGGGTTTCATCTTTCTTCT-3′, 5′-AGAAGAAAGATGAAACC CCTATCGCCACTGGGATTGTGGG-3′, 5′-CTCGAGTGCTCCACTAAACTCACTGCTTG GCGGTG CCA-3′, 5′-CTCGAGATGCGACCCTCAGGGACTGCGAGAACCAAGC-3′, 5′-ACAGCCACAGAGA CCCCAAAGGATGGGATCTTTGGCCCT T-3′, 5′-AAGGGCCAAAGATCCCATCCTTTGGGGTC TCTGTGGCTGT-3′, 5′-CTCGAGGCCCAGAACGTCCAGGTAACTGGGTGGC-3′, 5′-AAGCTGA CGTCGACGGAGCACAAGAAGGAGGG-3′, 5′-CCCTCCTTCTTGTGCTCCGAAGGCGTCACAT TGTCCG-3′, 5′-CTCCTCTGTCCACATTTGTTCATGAAGAGGCCGATCCCAA-3′, 5′-TTGGGAT CGGCCTCTTCATGAACAAATGTGGACAGAGGAG-3′.

4.7. DNA transfection

CHO-K1 cells seeded in 24-well plate (>80% confluent) were transiently transfected with various plasmids (0.8 μg/well), for 18–24 h by Lipofectamine™2000 (1.5 μl/well, Invitrogen), according to manufacturer's instruction.

4.8. Statistical analysis

The results shown are from at least three independent experiments. Data are expressed as the means ± standard deviations (SD). Data were analyzed for statistical significance using one-way ANOVA, and differences were considered significant at $p < 0.05$ as indicated.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2013.05.003.

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