論文の内容の要旨

応用生命工学専攻
平成 22 年度博士課程入学
氏　　名　　烏漢其木格
指導教員名　　北本　勝ひこ

論文題目

A study on the mechanism whereby lysophosphatidylcholine enhances neurotrophin-induced signals

（神経栄養因子シグナルに対するリゾホスファチジルコリンの増強作用に関する研究）

Introduction

Neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are essential regulators of neuronal differentiation, survival, plasticity, and other associated physiological actions of neurons throughout the entire life. By binding to their receptors TrkA (for NGF), TrkB (for BDNF), and p75 (for both), NGF and BDNF activate avariety of signals such as Ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase-Akt, and phospholipase Cγ pathways. Our research group previously demonstrated that secretory phospholipase A_2 ($sPLA_2$), a group of enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of membrane phospholipids to release free fatty acids and lysophospholipids, shows neurotrophin-like activity, i.e. induction of neurite outgrowth in PC12 cells, and protection of cerebellar granule neurons (CGNs) from apoptosis, mimicking the actions of NGF and BDNF. Subsequent studies showed that the neurotrophin-like actions of $sPLA_2$ are mediated through the release of one of the lysophospholipids, lysophosphatidylcholine (LPC). Indeed, LPC added to the cultures of PC12 and CGNs recapitulated the neurotrophin-like activity of $sPLA_2$. In this study, I have demonstrated that LPC promotes neurotrophin-Trk receptor signals in PC12 cells and in CGNs. I have also analyzed the underlying mechanism and confirmed that LPC specifically enhances NGF-induced signals through the extracellular domain of TrkA in PC12 cells, and BNDF-induced signals through TrkB in CGNs. Results from the neurite outgrowth assay in PC12 cells suggested that LPC at least partially potentiates NGF-induced differentiation of PC12 cells. It was unlikely that these processes were mediated by G

protein-coupled receptors G2A and GPR4, although they have been implicated in the biological actions of LPC in previous studies. Taken together, the findings obtained in this study might provide important evidences for the investigation of the mechanism by which LPC displays neurotrophin-like effect.

Chapter 1. Lysophosphatidylcholine promotes NGF-induced MAPK and Akt signals by enhancing the activation of TrkA in PC12 cells

LPC is one of the major lysophospholipids generated from the hydrolysis of phosphatidylcholine (PC) by $sPLA_2$. In our previous studies, LPC generated by $sPLA_2$ was found to induce neurite outgrowth in PC12 cells. Since NGF also induces neuronal differentiation, I tested if a cross-talk between NGF- and LPC-induced signals exists in PC12 cells by examining the effect of LPC on NGF-induced MAPK and Akt phosphorylation. I found that LPC significantly enhances NGF-induced MAPK and Akt phosphorylation. Other lysophospholipids, such as lysophosphatidic acid, lysophosphatidylethanolamine, and lysophosphatidylserine, did not display similar effect. Quantitative RT-PCR analysis showed that LPC upregulates the expression level of NGF-induced immediate early genes, c-*fos* and *NGF-IA*, which are necessary for the initiation and maintenance of differentiation. Neurite outgrowth assay showed that LPC partially potentiates NGF-induced differentiation of PC12 cells. Next, the signaling pathway by which LPC potentiates NGF-induced MAPK and Akt phosphorylation in PC12 cells was characterized. Phosphorylation of both MEK and TrkA, the upstream cellular components of MAPK, was enhanced by LPC. In contrast, LPC did not show any effect on MAPK phosphorylation induced by other growth factors, epidermal growth factor (EGF) and basic fibroblast growth factor. In accordance, EGF receptor phosphorylation induced by EGF was not increased by LPC. Furthermore, Akt phosphorylation induced by insulin-like growth factor-1 (IGF-1) was not affected by LPC. Collectively, these results indicate that LPC specifically promotes NGF-induced MAPK and Akt phosphorylation through enhancing the activation of TrkA.

Chapter 2. Lysophosphatidylcholine potentiates BDNF-induced MAPK and Akt signals through stimulating the activation of TrkB in cerebellar granule neurons

BDNF plays critical roles in regulating the survival and functions of neurons, particularly those in the brain, through binding to its receptor TrkB. BDNF is known to support the survival of cerebellar granule neurons (CGNs), the most abundant neurons in the brain, through the activation of Ras-MAPK pathway. Cultured CGNs are suitable model for studying the cell survival, since they undergo apoptosis when shifted to the culture medium containing low concentration of potassium (LK). IGF-1 is also known to protect CGNs from LK-induced

apoptosis through PI3K-Akt cascade. Previously, our research group showed that LPC protects CGNs from LK-induced apoptosis, mimicking the actions of BDNF and IGF-1, although the mechanism has not been identified. In this Chapter, I studied the effect of LPC on BDNF-induced MAPK and Akt phosphorylation in TrkB-transfected CHO-K1 cells and in CGNs. I first confirmed that MAPK was not phosphorylated upon treatments with BDNF, LPC, or both, in the wild type and vector-transfected CHO-K1 cells. In TrkB-transfected CHO-K1 cells, BDNF slightly induced phosphorylation of MAPK, which was significantly enhanced by LPC. In CGNs, although LPC alone did not induce phosphorylation of MAPK and Akt, it significantly elevated phosphorylation of MAPK and Akt induced by BDNF, but not by IGF-1. Furthermore, BDNF-induced TrkB phosphorylation was increased by LPC, suggesting that LPC promotes BDNF-induced MAPK and Akt signals through enhancing the activation of TrkB. Although LPC failed to further potentiate the effect of BDNF in rescuing CGNs from apoptosis, the results presented here, together with those shown in Chapter 1, indicate that LPC specifically enhances neurotrophin-Trk receptor signaling cascades.

Chapter 3. Analyses of the role of lysophosphatidylcholine on NGF-induced TrkA signal

Results shown in Chapter 1 demonstrated that TrkA, but not EGFR, is responsive to the effect of LPC. To further understand the mode of action of LPC on TrkA, I aimed to analyze the domain(s) of TrkA involved in the effect of LPC. To this end, I first analyzed receptor phosphorylation in TrkA-, EGFR-, or TrkA/EGFR chimera-transfected cells. However, spontaneous phosphorylation of TrkA occurred in transfected cells, and this was not further increased by NGF and/or LPC treatments, which hampered further analysis.

I next tested the effect of NGF, EGF, and LPC on the downstream signal, MAPK phosphorylation, in the same experimental system. I found that the wild type and vector-transfected CHO-K1 cells do not respond to NGF and EGF, i.e. MAPK was not phosphorylated upon NGF and EGF treatments, since no functional TrkA and EGFR were expressed in CHO-K1 cells. In TrkA-transfected cells, NGF weakly induced MAPK phosphorylation, and it was significantly increased by LPC. In EGFR-transfected cells, EGF induced MAPK phosphorylation, but this was not affected by LPC. These results indicate that transfected TrkA and EGFR behaved as those in PC12 cells. Next, the domain(s) of TrkA involved in the effect of LPC was analyzed by examining MAPK phosphorylation in TrkA/EGFR chimera-transfected CHO-K1 cells. TrkA/EGFR chimeras C1, C2, C3, and C4 were constructed by swapping the extracellular (ED), transmembrane (TMD), and intracellular (ID) domains between TrkA and EGFR. In C1 (TrkA ED/EGFR TMD+ID chimera)- or C3 (TrkA ED+TMD/EGFR ID chimera)-transfected cells, LPC enhanced MAPK phosphorylation triggered by NGF, as was seen in the TrkA-transfected cells. In C2 (EGFR ED/TrkA TMD+ID

chimera)- or C4 (EGFR ED+TMD/TrkA ID chimera)-transfected cells, MAPK was strongly phosphorylated upon EGF treatment, but this was not further enhanced by LPC. These results indicate that the ED, but not TMD and ID, of TrkA is responsible for the effect of LPC in enhancing NGF-induced MAPK phosphorylation.

As described in Introduction, LPC is generated through the hydrolysis of PC by $sPLA_2$. I next examined the effect of $sPLA_2$ addition on NGF-induced MAPK phosphorylation. I found that exogenously-added $sPLA_2$ enhances NGF-induced MAPK phosphorylation at a comparable level to LPC. This suggests that LPC generated *in situ* by the hydrolysis of plasma membrane PC acts synergistically with NGF.

Accumulating evidence suggests the involvement of G protein-coupled receptor, G2A and GPR4, in the biological actions of LPC. In addition, our research group previously found that $sPLA_2$-induced neuritogenesis in PC12 cells was mediated by G2A. I therefore examined if G2A and GPR4 regulate the effect of LPC on NGF-induced MAPK phosphorylation in PC12 cells. However, overexpression of neither G2A nor GPR4 affected the enhancement of NGF-induced MAPK phosphorylation by LPC, indicating that the action of LPC on NGF-induced MAPK phosphorylation is not mediated by G2A and GPR4.

It is well accepted that NGF induces dimerization and autophosphorylation of TrkA, thereby activating the downstream signaling events. Recently 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. To examine if LPC regulates the dimerization state of TrkA, I performed crosslinking experiment using the divalent crosslinker bis[sulfosuccinimidyl] suberate In accordance with the study, I succeeded in detecting TrkA dimer in TrkA-transfected PC12 cells irrespective of NGF addition. No increase in the amount of TrkA dimer was detected by LPC addition, indicating that LPC does not affect dimerization of TrkA.

Conclusion

Although various biological activities have been attributed to LPC, the precise mechanism is not fully understood. Results in this study have demonstrated that LPC enhances NGF-induced MAPK and Akt signaling pathways via the extracellular domain of TrkA. Similar effect was observed in BDNF-TrkB signaling. Further analyses suggested that LPC might display neurotrophin-like effect either by promoting NGF-induced MAPK and Akt signaling cascades, or through activating additional signaling pathway independently of NGF-TrkA. Deciphering the molecular mechanism of action of LPC might provide an important clue for the development of a new therapeutic method for neurodegenerative diseases.