Identification of Lysophosphatidic Acid Receptor 1 in Astroglial Cells as a Target for Glial Cell Line-derived Neurotrophic Factor Expression Induced by Antidepressants

Naoto Kajitani, Kenako Miyano, Mami Okada-Tsuchioka, Hiromi Abe, Kei Itagaki, Kazue Hisaoka-Nakashima, Norimitsu Morioka, Yasuhiro Uezono, and Minoru Takebayashi

From the Division of Psychiatry and Neuroscience, Institute for Clinical Research, National Hospital Organization (NHO) Kure Medical Center and Chugoku Cancer Center, 3-1 Aoyama, Kure 737-0023, the Division of Cancer Pathophysiology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, the Department of Psychiatry, National Hospital Organization (NHO) Kure Medical Center and Chugoku Cancer Center, 3-1 Aoyama, Kure 737-0023, the Department of Pharmacology, Hiroshima University Graduate School of Biomedical & Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, and the Division of Supportive Care Research, Exploratory Oncology Research & Clinical Trial Center, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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Preclinical and clinical evidence suggests that glial cell line-derived neurotrophic factor (GDNF) is important in the therapeutic effect of antidepressants. A previous study demonstrated that the tricyclic antidepressant amitriptyline induces Gαq/11 activation, which leads to GDNF expression in astrocytes. However, the specific target expressed in astrocytes that mediates antidepressant-evoked Gαq/11 activation has yet to be identified. Thus, the current study explored the possibility that antidepressant-induced Gαq/11 activation depends on lysophosphatidic acid receptor 1 (LPAR1), a Gq/11-coupled receptor. GDNF mRNA expression was examined using real-time PCR and Gαq/11 activation was examined using the cell-based receptor assay system CellKeyTM in rat C6 astroglial cells and rat primary cultured astrocytes. LPAR1 antagonists blocked GDNF mRNA expression and Gαq/11 activation evoked by various classes of antidepressants (amitriptyline, nortriptyline, mianserin, and fluoxetine). In addition, deletion of LPAR1 by RNAi suppressed amitriptyline-evoked GDNF mRNA expression. Treatment of astroglial cells with the endogenous LPAR agonist LPA increased GDNF mRNA expression through LPAR1, whereas treatment of primary cultured neurons with LPA failed to affect GDNF mRNA expression. Astrocytic GDNF expression evoked by either amitriptyline or LPA utilized, in part, transactivation of fibroblast growth factor receptor and a subsequent ERK cascade. The current results suggest that LPAR1 is a novel, specific target of antidepressants that leads to GDNF expression in astrocytes.

In the central nervous system, glial cells, especially astrocytes, are believed to play a critical role in the pathophysiology of major depressive disorder and possible targets of clinically used antidepressant medications (1). One of the major roles of astrocytes is the production of neurotrophic/growth factors, which are crucial in the process of neural plasticity (2). Recently, both clinical and preclinical studies have demonstrated that increased production of glial cell line-derived neurotrophic factor (GDNF) upon treatment of antidepressants is believed to play an important role in the therapeutic effect of antidepressants (3–5).

Previous studies have demonstrated that classes of antidepressants increase GDNF mRNA expression and release in rat C6 astroglial cells (C6 cells) and rat primary cultured astrocytes (primary astrocytes), but not in primary neurons (6, 7). Furthermore, the tricyclic antidepressant amitriptyline induced the Gαq/11 activation in astroglial cells, leading to GDNF expression through a monoamine-independent mechanism, possibly via a transactivation-like cascade of fibroblast growth factor receptor (FGFR)/FGFR substrate 2α (FRS2α)/ERK (8, 9). These results suggest the possibility that astrocytes have a unique Gαq/11 activation site that is responsive to antidepressants. However, the specific target expressed in astrocytes that mediates the amitriptyline-evoked Gαq/11 activation has yet to be identified.

Lysophosphatidic acid receptor 1 (LPAR1), a Gq/11-coupled receptor abundantly expressed in the brain, has been shown to be involved in neurological and psychiatric disorders (10–12). In CHO-K1 fibroblasts, antidepressants induced phosphorylation of ERK1/2 through LPAR1 (13). However, whether LPAR1 is involved in antidepressant-evoked signaling in cells of the central nervous system is unknown. Therefore, the current study examined the possibility that LPAR1 expressed on astro-
cytes is the G\textsubscript{i/o}-coupled receptor that mediates GDNF expression following antidepressant treatment.

**Results**

Several Different Classes of Antidepressants Increase the GDNF mRNA Expression and G\textsubscript{i/o} Activation through LPAR1 in C6 Cells—To test whether LPAR1 is involved in antidepressant-evoked GDNF expression, C6 cells were incubated with LPAR antagonists. Treatment with the various antidepressants, including tricyclic antidepressants (amitriptyline and nortriptyline), a tetracyclic antidepressant (mianserin), and a selective serotonin reuptake inhibitor (fluoxetine), significantly increased GDNF mRNA expression (Fig. 1A). The antidepressant-evoked increases of GDNF mRNA expression were significantly blocked by AM966 (a selective LPAR1 antagonist) and Ki16425 (a selective LPAR1/3 antagonist), but not H2L5186303 (a selective LPAR2 antagonist) (Fig. 1A). Incubation of C6 cells with LPAR antagonists alone did not significantly affect GDNF mRNA expression (data not shown).

An electrical impedance-based biosensor (CellKey™ assay) is specifically tailored to G protein-coupled receptor detection and can distinguish signals between the G\textsubscript{s}, G\textsubscript{i/o}, and G\textsubscript{q} subfamilies measured by cell layer impedance (\(\Delta Z\)) (8, 14). Amitriptyline increased \(\Delta Z\), indicative of G\textsubscript{i/o} activation. This
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increase of ΔZ was reduced by pertussis toxin (PTX, a specific Gαi/o inhibitor) (Fig. 1B), which confirms a previous finding (8). Addition of LPAR1 antagonists, either AM966 or Ki16425, also reduced the amitriptyline-induced increase of ΔZ (Fig. 1B). The effects of blocking LPAR1 and LPAR1/3 were comparable with that obtained with PTX. The effect of the inhibitors on the increase of ΔZ evoked by amitriptyline was statistically significant (PTX, p < 0.01; AM966, p < 0.01; and Ki16425, p < 0.05). Other antidepressants (nortriptyline, mianserin, and fluoxetine) also increased ΔZ, which was attenuated by AM966 (Fig. 1C). The inhibitory effects of AM966 were significant (nortriptyline, p < 0.05; mianserin, p < 0.01; and fluoxetine, p < 0.05). By contrast, non-antidepressant drugs, haloperidol and diazepam, did not affect either GDNF expression (7, 15) or ΔZ (data not shown) in C6 cells.

Knockdown of LPAR1 Decreases the Amitriptyline-evoked GDNF mRNA Expression in C6 Cells—To further demonstrate that LPAR1 is involved in GDNF expression induced by antidepressants, C6 cells were transfected with LPAR siRNA. LPAR1 consists of an unmodified form (41 kDa) and a glycosylated form (50–75 kDa) (16). Transfection with LPAR siRNA, but not negative control siRNA, significantly decreased protein levels of LPAR1 to less than one-third of vehicle (Fig. 2A). Amitriptyline-evoked GDNF mRNA expression was significantly suppressed by LPAR1 siRNA transfection, whereas transfection with negative control siRNA did not significantly affect the amitriptyline-evoked GDNF mRNA expression (Fig. 2B). Transfection with LPAR1 siRNA or negative control siRNA alone did not significantly affect GDNF mRNA expression (data not shown). By contrast, transfection of C6 cells with either LPAR2 or LPAR3 siRNA, which showed specific knockdown of the expression of their respective receptors, did not affect amitriptyline-evoked GDNF mRNA expression (supplemental Fig. S1).

Amitriptyline Increases GDNF Protein Release through LPAR1 in C6 Cells—Significant GDNF protein release from C6 cells was observed 48 h after amitriptyline treatment (15). However, because 48 h treatment with LPAR antagonists causes a non-specific toxic effect in C6 cells, the effect of LPAR antagonists on the amitriptyline-induced GDNF release at a shorter treatment time (24 h) was examined. Twenty-four h treatment of C6 cells with 25 μM amitriptyline did not significantly increase GDNF release (15), whereas 50 μM amitriptyline significantly increased GDNF release (Fig. 2C). This amitriptyline-induced GDNF release was significantly inhibited by AM966 and Ki16425, but not H2L5186303 (Fig. 2C).

Treatment with LPA Increases GDNF mRNA Expression through LPAR1 in Astroglial Cells but Not in Neurons—C6 cells were treated with LPA (100 nM), an endogenous LPAR ligand, and GDNF mRNA expression was quantified. Significant GDNF mRNA expression was observed 1 h after LPA treatment and returned to basal levels 6 h after the beginning of treatment (Fig. 2D) in a concentration-dependent manner (EC_{50} = 61.99 nm). Significant GDNF protein release was also observed with 24 h treatment of LPA in C6 cells (control, 59.3 ± 8.0; 100 nM, 83.2 ± 4.2; 1 μM, 100.1 ± 5.2 pg/ml, respectively; n = 4), which was comparable with the increase observed with 48 h incubation of 25 μM amitriptyline (8). Furthermore, pre-treatment with PTX, AM966, or Ki16425, but not H2L5186303, significantly blocked the GDNF mRNA expression evoked by LPA (Fig. 2E).

Amitriptyline treatment increases GDNF mRNA expression in primary astrocytes as well as C6 cells but not in primary neurons (6). Thus, the effect of LPA on GDNF mRNA expression in primary astrocytes and neurons were examined. Treatment of primary astrocytes with LPA significantly increased GDNF mRNA expression, which was significantly attenuated with PTX, AM966, or Ki16425, but not H2L5186303 (supplemental Fig. S2A). However, treatment of primary neurons with LPA (1 μM) failed to affect the GDNF mRNA expression (supplemental Fig. S2B).

Activation of LPAR1 Evoked by Either Amitriptyline or LPA Induces the Phosphorylation of FRS2α and ERK1/2 in C6 Cells—The potential involvement of the transactivation cascade (FGFR, FRS2α, and ERK1/2) related to GDNF expression (8, 9) in LPAR1-mediated signaling was examined. Preincubation with either SU5402 (an FGFR inhibitor) or U0126 (a MEK/ERK inhibitor) completely blocked LPA-evoked GDNF mRNA expression in C6 cells (Fig. 2F). Amitriptyline-induced phosphorylation of ERK1/2 and phosphorylation of FRS2α, a surrogate of FGFR activation (8), were significantly inhibited by AM966 and Ki16425, but not H2L5186303 (Fig. 2F). LPA-induced phosphorylation of both ERK1/2 and FRS2α were also significantly inhibited by AM966 and Ki16425 (Fig. 2G).

Discussion

The current data are the first to clearly demonstrate that Gα_i/o-coupled LPAR1 in astrocytes mediates GDNF mRNA expression evoked by various antidepressants. It has been previously suggested that the FGFR/FRS2α/ERK cascade mediates, in part, amitriptyline-induced GDNF expression (8, 9). The current results demonstrated that a transactivation cascade, FRS2α and ERK1/2, observed with amitriptyline treatment is mediated through LPAR1. Furthermore, the mechanism is unique to LPAR1 expressed on astrocytes as LPA treatment of neurons did not increase GDNF mRNA expression. The current findings suggest a novel non-neural, astrocytic target of antidepressants.

In the current study, PTX decreased the amitriptyline-evoked increase of ΔZ (the pattern of Gα_i/o activation), indicating involvement of PTX-sensitive Gα_{i/o} protein. Addition of LPAR1 antagonists, either AM966 or Ki16425, also reduced the amitriptyline-induced increase of ΔZ to the extent observed when PTX was added to amitriptyline. LPAR1 is coupled to a PTX-sensitive Gα_{i/o} in astrocytes (17). Therefore, these findings suggest that amitriptyline could increase ΔZ through LPAR1 coupled to a PTX-sensitive Gα_{i/o} protein. However, the inhibitory effect of PTX on the increase of ΔZ appears to be partial, suggesting the possibility that amitriptyline activates a signaling pathway that involves PTX-insensitive Gα proteins. In the Gα_{i/o} subfamily, Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_{i4}, and Gα_{i5} have been identified and share similar properties in signal transduction (18). Among these subunits, Gα_{i} lacks the C-terminal cysteine residue and is not inactivated by PTX treatment (19, 20). C6 cells express Gα_{i2}, Gα_{i3}, Gα_{i4}, Gα_{i5}, and Gα_{i} (8). On one hand, it is suggested that the amitriptyline-
FIGURE 2. The involvement of LPAR1 in amitriptyline- and LPA-evoked GDNF expression, ERK1/2, and FRS2α in C6 cells. A, effect of LPAR1 siRNA on LPAR1 protein levels. Cells were transfected with vehicle, LPAR1 siRNA, or negative control (Nega) siRNA for 48 h. LPAR1 proteins were quantified by immunoblotting. Immunoblots from a representative experiment are shown. The numbers under the immunoblots indicate mean ± S.E. relative-fold change in expression compared with vehicle. **, p < 0.01; n.s., not statistically significant (Bonferroni’s test; n = 4–5). B, effect of LPAR1 siRNA on amitriptyline-evoked GDNF mRNA expression. Cells were transfected with vehicle, LPAR1 siRNA, or Nega siRNA for 48 h and subsequently treated with 25 μM amitriptyline for 3 h. **, p < 0.01; +, p < 0.05; n.s., not statistically significant (Bonferroni’s test; n = 6–16). C, the effect of LPAR antagonists on the amitriptyline-GDNF release. C6 cells were pretreatment with either AM966 (AM), K16425 (Ki), or H2L5186303 (H2L) for 0.5 h and subsequently treated with 50 μM amitriptyline for 24 h. **, p < 0.01; +, p < 0.05; ++, p < 0.01; n.s., not statistically significant (Bonferroni’s test; n = 8–10). D, time-dependent effect of LPA on GDNF mRNA expression. Cells were treated with 100 nM LPA for the indicated period in hours. *, p < 0.05; **, p < 0.01 versus control (Bonferroni’s test; n = 3–7). E, effect of LPAR-related inhibitors on LPA-evoked GDNF mRNA expression. Cells were pretreated with either PTX for 3 h, or AM, Ki, H2L, SU5402 (SU), or U0126 (U) for 0.5 h and subsequently treated with 100 nM LPA for 1 h. **, p < 0.01 versus control, and ++, p < 0.01 versus LPA alone (Bonferroni’s test; n = 3–4). F and G, effect of LPAR antagonists on ERK1/2 phosphorylation and FRS2α phosphorylation evoked by either amitriptyline (P) or LPA (G). Cells were pretreated with AM, Ki, or H2L for 0.5 h and subsequently treated with either 25 μM amitriptyline for 5 min or 100 nM LPA for 2 min. Phosphorylated/total ERK1/2 and phosphorylated/total FRS2α were quantified by immunoblotting. Immunoblots from a representative experiment are shown on the left and quantitative data are shown on the right. *, p < 0.05; **, p < 0.01 versus control; and +, p < 0.05; ++, p < 0.01 versus amitriptyline or LPA alone (Bonferroni’s test; n = 3–8).
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induced increase of ΔZ involves both PTX-sensitive Go subunits, such as Go, and Go, and PTX-insensitive subunits, such as Go. On the other hand, the contribution of PTX-insensitive subunits may not be significant, as amitriptyline-induced GDNF mRNA expression is completely suppressed by PTX (8). Therefore, amitriptyline-induced GDNF expression involves the activation of LPAR1 coupled to a PTX-sensitive Go in C6 cells.

LPAR1 inhibition significantly blocked both mRNA expression and protein release of GDNF evoked by amitriptyline in C6 cells. A partial inhibitory effect of LPAR1 antagonists on GDNF release evoked by amitriptyline was observed, suggesting the possibility that amitriptyline increases GDNF release via a LPAR1-dependent and -independent manner. A previous study showed a case that GDNF protein release was regulated by a mechanism that did not alter GDNF mRNA levels (21), suggesting a possible role of post-transcriptional regulation. Therefore, amitriptyline increases GDNF release through LPAR1-mediated GDNF mRNA induction and possibly through other post-transcriptional regulation pathways.

LPAR1 is expressed in several types of cells in the brain, especially glial cells, in various stages of the life cycle. LPAR1 is involved in myelination, proliferation, and maturation of neurons (11). Primary astrocytes used in the current study showed basal levels of LPAR1 mRNA that were 45 times higher than that of primary neurons. Thus, the lack of effects of amitriptyline and LPA on GDNF expression in primary neurons (6) could be due to their low level of LPAR1 expression. A previous study showed that factors released from astrocytes following LPA treatment, but not LPA itself, promoted neuronal differentiation (22). Therefore, it is possible that antidepressant treatment, through activation of astrocytic LPAR1, induces the release of a number of factors, including GDNF, which promote neural plasticity.

Previous in vivo studies have shown that behavioral response to antidepressants involves increasing GDNF expression and PTX-sensitive signaling in the brain. Chronic stress in mice led to depressed-like behavior and decreased brain expression of GDNF, both of which were reversed with antidepressant treatment (5). Administration of PTX completely blocked the pharmacological effect of amitriptyline in mice as assessed in the forced swim test (23). In vivo studies will be needed to verify if astrocytic LPAR1 is involved in the behavioral response to antidepressant.

It is currently unclear whether antidepressants directly or indirectly induce LPAR1 activation. The CellKey™ assay showed that antidepressants rapidly induced Go, activation, indicating that antidepressants directly activate LPAR1 in astroglial cells. A previous study reported that several classes of antidepressants accumulate in the lipid rafts formed on the plasma membrane (24). Lipid rafts serve as a signaling platform for G protein-coupled receptor clustering, including LPAR1 (25, 26). Thus, it is possible that antidepressants interact with LPAR1 in the lipid raft microdomains of cells. Identifying LPAR1 as an antidepressant binding site could be the next step to lead to the development of novel antidepressants based on LPAR1 activation.

Experimental Procedures

All experimental procedures were performed according to the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Institutional Animal Care and Use Committee of Hiroshima University, and followed the Guideline for Animal Experiments for National Hospital Organization Kure Medical Center and Chugoku Cancer Center.

Reagents—Reagents were obtained from the following sources: amitriptyline and mianserin (Wako Pure Chemicals, Osaka, Japan); SU5402 (Merck, Darmstadt, Germany); fluoxetine and nortriptyline (Sigma); PTX and U0126 (Calbiochem, San Diego, CA); 1-oleoyl LPA and Ki16425 (Cayman, MI); AM966 (Medchem Express, Monmouth Junction, NJ), and H2L5186303 (Tocris Bioscience, Minneapolis, MN).

Cell Culture and Drug Treatment—Preparation of C6 cells has been described previously (8). Primary astrocytes and neurons were prepared from 1-day-old neonatal Wistar rats, as described previously (27). Primary astrocytes in culture for 3 weeks were used. Primary neurons in culture for 2 weeks were used and were considered well differentiated, mature neurons (27).

The concentrations of amitriptyline (25 and 50 μM) used in this report are not toxic to C6 cells/primary astrocytes (7, 27). Antidepressants accumulate in the brain at concentrations severalfold higher than that in plasma, because of their high lipophilic properties (28, 29). In the current study, to replicate clinical brain concentrations and to standardize across antidepressants, all antidepressants were applied to the cells at a concentration of 25 μM.

To determine whether LPAR1 is involved in antidepressant-evoked GDNF expression and to characterize the transactivation pathway, cells were incubated with AM966 (1 μM), Ki16425 (1 μM), H2L5186303 (1 μM), PTX (100 ng/ml), SU5402 (25 μM), or U0126 (10 μM). Concentrations of these inhibitors were based on previous reports (8, 30), which were selective for their respective targets.

RNA Extraction and Real-time PCR Analysis—Total RNA was extracted from cells with a RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. First strand cDNA was synthesized from total RNA using PrimeScript RT Master Mix (Takara Bioscience, Shiga, Japan). Real-time PCR was performed with the Thermal Cycler Dice Real Time System II (Takara Bioscience), using TaqMan probes and primers for rat GDNF and GAPDH (Rn00569510_m1 and Rn99999916_s1, respectively; Applied Biosystems, Foster, CA).

CellKey™ Assay—The CellKey™ assay, a label-free cell-based assay that detects G-protein activation, has been described previously (8). Briefly, C6 cells were cultured on a standard CellKey™ 96-well microplate. Just before assay, cells were washed with assay buffer (Hanks’ balanced salt solution with 20 mM HEPES and 0.1% BSA) and allowed to equilibrate in the assay buffer for 30 min at 29 °C. The CellKey™ instrument applied small voltages every 10 s and measured the impedance of the cell layer (ΔZ). In this study, a 5-min baseline was recorded, drugs were added, and then ΔZ was measured for 10

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imm. The data were quantified using ΔZ at 10 min after drug injection.

**Immunoblotting Analysis**—The following antibodies were used: phospho-FRS2α (Tyr-196) (number 3864), p44/42 MAPK (Erk1/2) (number 9101), and phospho-p44/42 MAPK (Erk1/2) (number 4370) (Cell Signaling Technology, Beverly, MA); FRS2α (SNT1), (Sigma); EDG2 (LPAR1) (ab23698) (Abcam, Cambridge, MA); LPAR2 (sc25490) (Santa Cruz Bio-technology Inc., Santa Cruz, CA); and LPAR3 (ALR-033) (Alomone Labs., Jerusalem, Israel). Briefly, the proteins (20 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transblotted to PVDF membranes. The membranes were blocked with 5% (w/v) skim milk for 1 h at room temperature and incubated with the respective antibodies overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Chemiluminescent detection was performed using Immun-Star™ WesternC™ Kit (Bio-Rad), and the net intensities of each band were quantified using ChemiDoc™ XR+. (Bio-Rad) (27).

**Small Interfering RNA**—Small interfering RNA (8) were used to suppress expression of LPAR1, LPAR2, and LPAR3 in C6 cells. The cells were transfected with either 20 nm siRNA targeting rat LPAR1, LPAR2, LPAR3, or non-targeting siRNA (siGENOME, GE Healthcare) by using RNAiMAX (Life Technologies) according to the manufacturer’s instructions.

**Enzyme-linked Immunosorbent Assay**—GDNF release assay has been described previously (8). Briefly, C6 cells were cultured on 12-well plates. After drug treatment, the concentrations of GDNF protein in cell-conditioned media were determined using a GDNF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer’s instructions.

**Statistical Analysis**—Data are expressed as the mean ± S.E. of at least three independent experiments. Statistically significant differences among the means were determined using one-way analysis of variance with pairwise comparison carried out by Bonferroni’s method. Differences between two groups were statistically analyzed with Student’s t test. p values at less than 0.05 were taken as statistically significant.

**Author Contributions**—N. K., K. M., and M. O. T. conducted the experiments, analyzed the results, and wrote the initial draft of the manuscript. H. A. and K. I. performed the experimental support, analyzed the data, and prepared the figures. K. H. N. and N. M. prepared rat primary cultured astrocytes and neurons and conducted the experiments (supplemental Fig. S2). Y. U. and M. T. designed the experiments and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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