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Lysophosphatidylglycerol stimulates chemotactic migration in human natural killer cells

Seong Ho Jo [a], Sang Doo Kim [a], Jung Mo Kim [a], Ha Young Lee [a], Sun Young Lee [a], Jae Woong Shim [a], Jeanho Yun [a], Dong-Soon Im [b], Yoe-Sik Bae [a,*]

[a] Department of Biochemistry, College of Medicine, Dong-A University, 3-1 Dongdaesindong Seogu, Busan 602-714, Republic of Korea
[b] College of Pharmacy, Pusan National University, San 30 Jangjundong Geumjeonggu, Busan 609-735, Republic of Korea

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ABSTRACT

We observed that lysophosphatidylglycerol (LPG) stimulates chemotactic migration in human natural killer (NK) cells. The LPG-induced chemotactic migration of NK cells was completely inhibited by pertussis toxin (PTX). LPG also stimulated the extracellular signal-regulated kinase (ERK) and Akt activities in NK cells. LPG-stimulated ERK activity was inhibited by PTX, indicating the involvement of PTX-sensitive G-proteins. The preincubation of NK cells with an ERK inhibitor (PD98059) or phosphoinositide-3-kinase (PI3K) inhibitors (wortmannin and LY294002) completely inhibited LPG-induced chemotactic migration, suggesting the essential role of ERK and PI3K in the process. Moreover, LPG-induced chemotactic migration in NK cell was inhibited by Ki16425, an $LPA_{1/3}$ receptor-selective antagonist, suggesting the involvement of the Ki16425-sensitive G-protein coupled receptor (GPCR) in the process. Taken together, the results indicate that LPG stimulates chemotactic migration in NK cells through GPCR, suggesting a new function of LPG as a modulator of NK cell functioning.

Since natural killer (NK) cells play an important role in the regulation of immune responses, including anti-infective activity and anti-tumor activity, they have been associatively regarded as important factors in the treatment of cancer and infectious diseases [1,2]. The chemotactic migration of NK cells toward the infected or tumor cell-localizing sites is a crucial step for the initiation of immune responses. Even though certain chemokines have been found to regulate the chemotactic migration of NK cells, the extracellular signals that regulate NK the cell migration and lysis activity have not been fully identified.

Some lysophospholipids, including lysophosphatidic acid (LPA), have cytokine-like properties. In the case of LPA, reports have indicated that it induces cellular proliferation, migration and invasion in fibroblasts or some cancer cells, such as ovarian cancer cells [3,4]. Moreover, five G-protein coupled receptors (GPCR) located on the plasma membrane, including LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5, have been reported as target receptors for LPA [5–8]. Recently, LPG has also been reported to possess biological activity [9–11]. A previous report indicated that LPG prevents the binding of LPA into a putative LPA receptor on the cell-surface of mouse NIE-115 neuroblastoma cells [9]. Very recently, we reported that LPG stimulates an intracellular calcium increase, ERK, and Akt in human ovarian cancer cells [10]. LPG also stimulates the chemotactic migration and tube formation in human umbilical vein endothelial cells (HUVECs) [11]. In both studies, we suggested that LPG utilizes GPCRs other than the known LPA receptors, by showing no response to LPG in each LPA receptor expressing HepG2 cells and no blockage of LPG response with the selective LPA receptor antagonist, Ki16425. Since the studies pertaining to the biological role of LPG are very limited and the role of LPG in NK cells has not been examined, we investigated the effect of LPG on NK cell activity as well as the signaling pathway involved in this process.

Materials and methods

Reagents. 1-Acyl-2-hydroxy-*sn*-glycero-3-phospho-glycerol (LPG) and Na^+ lysophosphatate (LPA) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Pertussis toxin (PTX), PD98059, wortmannin, and LY294002 were purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagents from Amersham Biosciences (Piscataway, NJ), Phospho-extracellular signal-regulated protein kinase (ERK)1/2 and ERK2 antibodies were purchased from New England Biolabs (Beverly, MA). Phospho-Akt antibody was from Sigma–Aldrich (St. Louis, MO). The horseradish peroxidase-conjugated antibodies, which were converted to rabbit IgG were purchased from Kirkegaard & Perry, Inc. (Gaithersburg, MD).

The isolation of NK cells from peripheral blood. Peripheral blood was collected from healthy donors, and the human peripheral mononuclear cells were isolated by a lymphocyte separation

* Corresponding author. Fax: +82 51 241 6940.
E-mail address: yoesik@donga.ac.kr (Y.-S. Bae).

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medium gradient, as described previously [12]. Peripheral blood NK cells were isolated using a blood NK cell isolation kit (Miltenyi Biotec Order No. 130-092-657) from peripheral mononuclear cells. Briefly, non-NK cells (i.e., T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells), were magnetically labeled and depleted using a cocktail of biotin-conjugated antibodies and the NK Cell Microbead Cocktail according to the manufacturer's instructions (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The purity of isolated NK cells was analyzed by way of a FACS analysis using antibodies against CD3 and CD56. The resultant cocktail consists of greater than 97% purified $CD56^{+}CD3^{-}$.

Chemotaxis assay. Chemotaxis assays were performed in multiwell chambers (Neuroprobe Inc. Gaithersburg, MD) as previously described [13]. Briefly, isolated human NK cells were suspended in RPMI at 1×10^6 cells/ml, and a 25 μl aliquot of this suspension was placed into the upper well of a chamber. Next, the aliquot was separated by a 5 μm polyhydrocarbon filter from the lipid-containing lower well. The migrated cells were comprised of stained cells with hematoxylin, which were subsequently counted in five randomly chosen high power fields (400×) [13].

Stimulation of NK cells with LPG for Western blot analysis. NK cells (2×10^6) were stimulated with 10 μM LPG for several lengths of time. After stimulation, the cells were washed with serum-free RPMI 1640 medium and lysed in lysis buffer (20 mM Hepes, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Detergent-insoluble materials were pelleted by centrifugation (12,000*g*, 15 min, at 4 °C), followed by the removal of the soluble supernatant fraction and storage at either −80 °C until subsequent use or used immediately. The protein concentrations in the lysates were determined using the Bradford protein assay reagent.

Electrophoresis and immunoblot analysis. Protein samples were prepared for electrophoresis and subsequently separated using a 10% SDS–polyacrylamide gel and buffer system described previously [13]. Following electrophoresis, the protein samples were blotted onto a nitrocellulose membrane, which were then blocked by incubation in TBST (Tris-buffered saline, 0.05% Tween 20) containing 5% non-fat dried milk. Next, the membranes were incubated with anti-phospho-ERK antibody, anti-phospho-Akt kinase antibody or anti-Akt antibody, and then washed with TBST. The antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system by incubating membranes with 1:5000 diluted goat anti-rabbit IgG antibody coupled with horseradish peroxidase.

Statistics. The results are expressed as means ± SE of the number of determinations indicated. Statistical significance of differences was determined by student *t*-test. Significance was accepted when $P < 0.05$.

Results

LPG induces chemotactic migration in NK cells

Since NK cell migration is important for the induction of immune responses against infectious antigens or tumor antigens [1,2], we tested the effect of LPG on NK cell migration. We found that LPG strongly stimulated chemotactic migration in NK cells (Fig. 1A). Moreover, LPG-induced NK cell migration was detected at 1–10 μM concentrations (Fig. 1A). The potency of the LPG-induced NK cell chemotactic migration is similar to the chemotactic migration induced by LPA (Fig. 1A). To distinguish between the LPG-induced chemotaxis and chemokinesis, we performed migration assays in the absence or presence of LPG in the upper wells of the chambers, as described previously [15]. The addition of

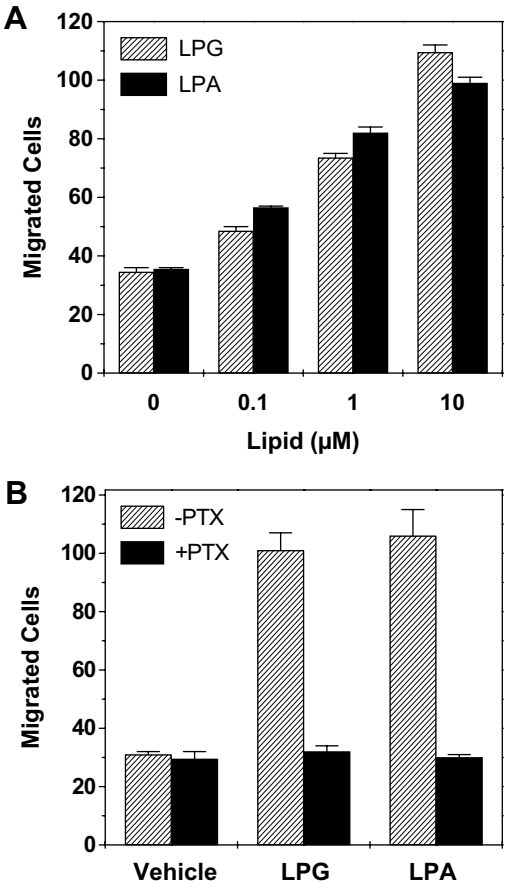

Fig. 1. LPG induces NK cell chemotaxis. Isolated human NK cells (1×10^6 cells/ml in serum-free RPMI) were used for the chemotaxis assay in the presence of several concentrations (0, 0.1, 1, and 10 μM) of LPG or LPA for 4 h at 37 °C (A). Isolated human NK cells were preincubated in the presence or absence of PTX (100 ng/ml) for 24 h prior to a chemotaxis assay using 10 μM of LPG or LPA (B). The number of cells that migrated was determined by counting 5 high power fields (400×). The data are expressed as means ± SE of three independent experiments performed in duplicate (A,B).

LPG (10 μM) in the upper chamber reduced the LPG-induced migration of NK cells to the lower well, indicating that LPG induces NK cell chemotaxis (data not shown).

Many chemoattractants including LPA have been reported to stimulate the chemotaxis of certain cells via PTX-sensitive G-proteins [14–16]. In our study, we tested the role of PTX-sensitive G-proteins on LPG-induced NK cell chemotaxis. As shown in Fig. 1B, the preincubation of NK cells with 100 ng/ml of PTX for 24 h prior to the chemotaxis assay, completely inhibited the LPG-induced NK cell chemotaxis. This contradicts the results observed with HUVECs. Moreover, the LPA-induced NK cell chemotaxis was also completely inhibited by PTX (Fig. 1B), indicating that LPG stimulates NK cell chemotaxis in a PTX-sensitive G-protein-dependent manner.

LPG-induced NK cell chemotaxis is mediated by ERK

Previous reports have demonstrated that ERK is involved in influencing the chemotactic migration of certain cell types [17,18]. In this study, we also investigated the effect of LPG on ERK activity in NK cells using a Western blot analysis. The stimulation of NK cells with 10 μM of LPG transiently stimulated ERK phosphorylation, which showed maximal activity at 5–10 min after stimulation (Fig. 2A). Since LPG-induced NK cell chemotaxis

was inhibited by PTX, we also examined the effect of PTX on LPG-induced ERK activity. The preincubation of NK cells with a 100 ng/ml concentration of PTX for 24 h prior to LPG stimulation almost completely inhibited LPG-induced ERK phosphorylation (Fig. 2B), suggesting that LPG stimulates ERK activity via the PTX-sensitive G-proteins. Next, we investigated whether the ERK pathway is required for LPG-induced NK cell chemotaxis. The preincubation of cells at several concentrations (0, 0.5, 5, and 50 μM) of PD98059 (a well-known MEK inhibitor) for 60 min at 37 °C prior to stimulation with LPG, was found to influence cellular chemotaxis (Fig. 2C), suggesting that LPG activates the ERK pathway and that this signaling is required for the LPG-induced chemotaxis of NK cells.

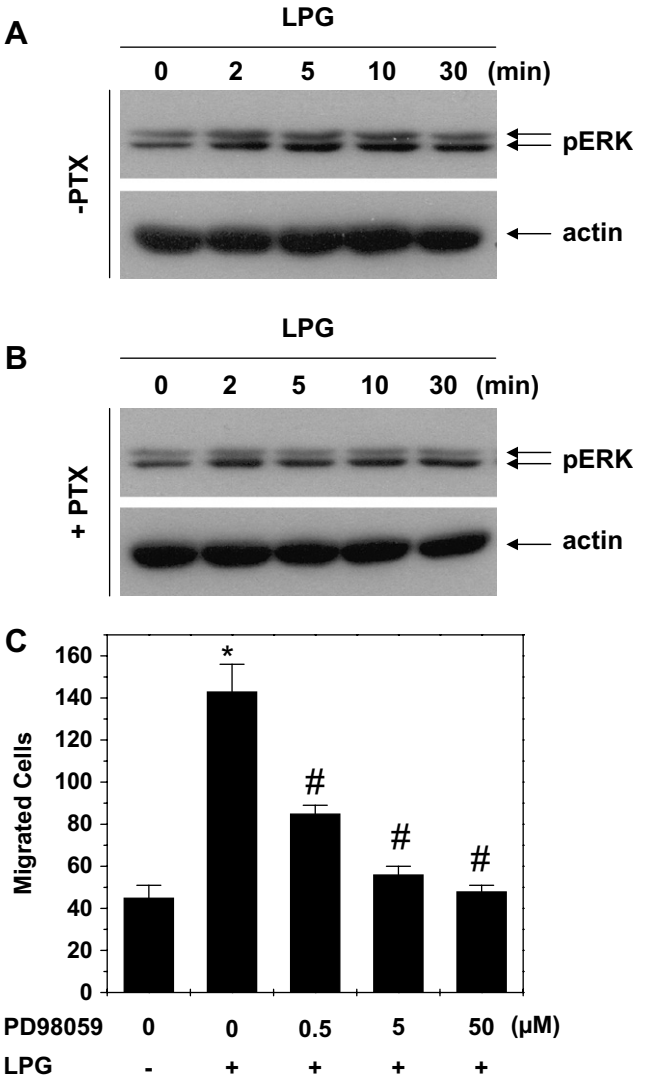

Fig. 2. LPG-induced NK cell chemotaxis is mediated ERK. Isolated human NK cells were preincubated in the absence (A) or presence (B) of PTX (100 ng/ml) for 24 h. Next, the cells were stimulated with 10 μM of LPG at various times (0, 2, 5, 10, and 30 min) (A). Each sample (30 μg of protein) was subjected to 10% SDS–PAGE, and phospho-ERK (pERK) was determined by immunoblotting using the anti-phospho-ERK antibody. The results shown are representative of at least three independent experiments (A,B). Isolated human NK cells were incubated in the presence of various concentrations of PD98059 (0, 0.5, 5, and 50 μM) for 60 min, prior to a chemotaxis assay, using 10 μM of LPG (C). The number of migrated cells was determined by counting in 5 high power fields (400×). The data are presented as means ± SE of three independent experiments performed in duplicate (C). *Significantly different from the control (vehicle) ($P < 0.05$). #Significantly different from the control (DMSO treated) ($P < 0.05$).

LPG stimulates Akt activity, resulting in chemotaxis

In the process of cell migration, phosphositide-3-kinase (PI3K)-mediated signaling has received much attention over the past decade [19,20]. Several reports have demonstrated that PI3K is involved in the chemotactic migration of leukocytes [21,22]. In this study, we examined the effect of LPG on the activity of Akt, a well-known downstream molecule of PI3K. LPG was found to transiently stimulate Akt phosphorylation in NK cells, revealing maximal activity at 5–10 min after stimulation (Fig. 3A). Next, we verified the role of the PI3K-mediated pathway on LPG-induced NK cell chemotaxis. As shown in Fig. 3B, the LPG-induced NK cell chemotaxis was almost completely inhibited by two different PI3K inhibitors (wortmannin and LY294002). The results indicate that LPG stimulates NK cell chemotaxis via PI3K-mediated signaling.

LPG-induced chemotactic migration is Ki16425-sensitive in NK cells

Since we observed that LPG stimulates chemotactic migration in a PTX-sensitive manner (Fig. 1), we examined the effect of an LPA receptor antagonist (Ki16425) on LPG-induced chemotaxis in NK cells. The preincubation of NK cells with 5 μM of Ki16425 (an LPA_1 and LPA_3-selective antagonist), inhibited the LPA-induced

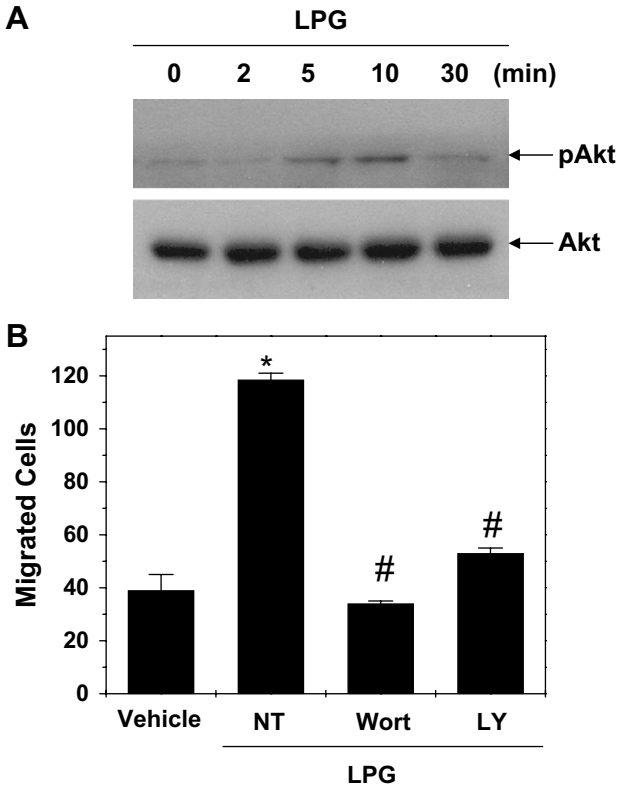

Fig. 3. LPG-induced NK cell chemotaxis is mediated PI3K signaling. Isolated human NK cells were stimulated with 10 μM of LPG at various times (0, 2, 5, 10, and 30 min) (A). Each sample (30 μg of protein) was subjected to 10% SDS–PAGE. The phospho-Akt (pAkt) was determined by immunoblotting via the anti-phospho-Akt antibody. The results shown are representative of at least three independent experiments (A). Isolated human NK cells were incubated with vehicle (DMSO), wortmannin (1 μM), or LY249002 (50 μM) for 15 min, prior to chemotaxis assay, using 10 μM of LPG (B). The number of migrated cells was determined by counting 5 high power fields (400×). The data are presented as means ± SE of three independent experiments performed in duplicate (B). *Significantly different from the control (vehicle) ($P < 0.05$). #Significantly different from the control (DMSO treated) ($P < 0.05$).

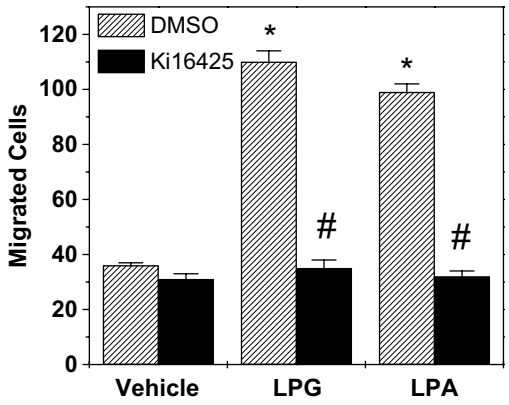

Fig. 4. LPG-induced NK cell chemotaxis is Ki16425-sensitive. Isolated NK cells were treated with vehicle (DMSO) or Ki16425 (5 μM) for 15 min, and then subjected to chemotaxis assays in the presence of 10 μM LPG or 10 μM LPA for 4 h. The number of migrated cells was determined by counting 5 high power fields (400×). The data are presented as means ± SE of three independent experiments performed in duplicate. *Significantly different from the control (vehicle) ($P < 0.05$). #Significantly different from the control (DMSO treated) ($P < 0.05$).

NK cell chemotaxis (Fig. 4). Moreover, the LPG-stimulated chemotaxis was also completely blocked by Ki16425 (Fig. 4). The results indicate that LPG stimulates chemotaxis in NK cells through a Ki16425-sensitive GPCR.

Discussion

The cytokine-like properties of lysophospholipids have been an important topic of study in past decades; especially since LPA and sphingosine-1-phosphate have been reported to regulate various biological responses [3–6,23,24]. In the case of LPG, we recently showed that it can act as a lipid ligand as well as stimulate some signaling molecules in ovarian cancer cells and HUVECs [10,11]. In this study, we report that LPG stimulates chemotactic migration in NK cells. We demonstrate that LPG stimulates NK cells, resulting in chemotactic migration. From these results, we suggest that LPG holds an important role in the initiation of immune responses against infectious microbes and tumor antigens. The anti-tumor activity initiated by NK cells has been associated with IFN-γ production [25]. We also tested the effect of LPG on IFN-γ production in NK cells. We found that LPG failed to stimulate IFN-γ production (data not shown). Also, LPG did not stimulate cytolytic activity in NK cells (data not shown). In summary, the results indicate that LPG regulates NK cell trafficking, but not anti-tumor activity. Moreover, the results suggest a new immune functional aspect of LPG as an NK cell chemoattractant.

In addition to the immunological LPG function in NK cells, two new findings regarding LPG signaling were demonstrated in the present study. In a previous report, we demonstrated that LPG-induced chemotactic migration in HUVECs was not inhibited by PTX, indicating that LPG modulates chemotactic migration independent of PTX-sensitive G-proteins in HUVECs. However, in this study, we found that the LPG-induced chemotaxis was completely inhibited by PTX (Fig. 1B). Such a different PTX sensitivity was also observed in ERK and Akt activation. The LPG-induced activation of ERK was sensitive to PTX in NK cells. However, ERK activation in HUVECs and ERK/Akt activation in OVCAR-3 human ovarian cancer cells were not affected by PTX treatment. This suggests two signaling pathways for the activation of the same ERK/Akt by LPG, depending on cell type. One signaling pathway occurs via the PTX-sensitive G-proteins, whereas the other occurs via the PTX-insensitive G-proteins. This may imply the presence of two subtypes of GPCRs for LPG or differential coupling of the same LPG receptor to different G-protein subtypes. Another interesting finding pertains to the sensitivity to Ki16425. In a previous report, we showed that LPG-stimulated chemotaxis in HUVECs was not inhibited by Ki16425, although LPA-induced chemotaxis were inhibited by Ki16425 [11]. In a separate experiment, we also demonstrated that LPG failed to stimulate NF-κB-driven luciferase activity in HepG2 cells (expressing LPA_1, LPA_2, or LPA_3 exogenously), although LPA stimulated the activity [10]. These results suggest that LPG may not act on Ki16425-sensitive LPA receptors such as LPA_1 or LPA_3. However, we found a contradictory result indicating that LPG-induced chemotactic migration was completely inhibited by Ki16425 in NK cells (Fig. 4), thereby implying that Ki16425-sensitive GPCRs are involved in the LPG action of NK cells. When considering the differential sensitivity to PTX in NK cells combined with the differential sensitivity to Ki16425, LPG seems to have indicate two GPCR subtypes; one being the Ki16425-insensitive GPCR, which is coupled to PTX-insensitive G-proteins and expressed in HUVECs and OVCAR-3 cells. The other is the Ki16425-sensitive GPCR, which is coupled to PTX-sensitive G-proteins and expressed in NK cells.

Another possible scenario to be considered is the Ki16425-sensitive LPG receptor in NK cells, which might be Ki16425-sensitive LPA receptors. Considering that LPG failed to stimulate the LPA_1, LPA_2, or LPA_3-transfected HepG2 cells, as well as the selectivity of Ki16425 on the $LPA_{1/3}$ receptors, such a possibility is very low. Therefore, LPG is considered to bind with a GPCR other than the LPA_1, LPA_2, LPA_3, or Ki16425-sensitive receptors. Because the sensitivity of LPA_4 and LPA_5 to Ki16425 has not yet been fully studied, in addition to the report of two new GPCRs functioning as LPA receptors, we were unable to exclude the possibility that the Ki16425-sensitive LPG receptor in NK cells is a LPA receptor, (except for $LPAs_{1-3}$), including LPA_4, LPA_5, GPR87, and P2Y5. Even in such a case, because the potency of LPG was similar to LPA, the putative GPCR should be considered to recognize LPG and LPA equally, excluding the partial agonist of LPG on the LPA receptor. Because reports on LPG-mediated cellular responses and the target receptor are so limited, further studies on the biological roles of LPG and on LPG-specific cell-surface receptor(s) are required. Our finding is expected to shed some light on the findings of LPG receptor(s) and the role(s) of LPG in future studies.

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