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Effect of lysophosphatidylglycerol on several signaling molecules in OVCAR-3 human ovarian cancer cells: Involvement of pertussis toxin-sensitive G-protein coupled receptor

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ABSTRACT

In this study, we observed that lysophosphatidylglycerol (LPG) stimulated intracellular calcium ($[Ca^{2+}]_i$) increase in OVCAR-3 human ovarian cancer cells. LPG-stimulated $[Ca^{2+}]_i$ increase was inhibited by U-73122 but not by U-73343, suggesting that LPG stimulates calcium signaling via phospholipase C activation. Moreover, pertussis toxin (PTX) almost completely inhibited $[Ca^{2+}]_i$ increase by LPG, indicating the activation of PTX-sensitive G-proteins. LPG-induced $[Ca^{2+}]_i$ increase was only observed in OVCAR-3 ovarian cancer cells and SK-OV3 ovarian cancer cells among tested several cell types. LPG also induced extracellular signal-regulated kinase (ERK) and Akt phosphorylation in OVCAR-3 ovarian cancer cells. Pertussis toxin did not affect the LPG-induced activation of ERK and Akt phosphorylation. We also found that LPG failed to stimulate NF-κB-driven luciferase activity in exogenously LPA_1, LPA_2, or LPA_3-transfected HepG2 cells. Taken together we suggest that LPG stimulates a membrane bound receptor which is different from well-known LPA receptors (LPA_1, LPA_2, and LPA_3), resulting in at least two different signaling cascades; one involves a pertussis toxin-sensitive and phospholipase C-dependent $[Ca^{2+}]_i$ increase, and the other involves a pertussis toxin-insensitive activation of ERK and Akt in ovarian cancer cells.

1. Introduction

Lysophospholipids including lysophosphatidic acid (LPA) have been regarded to act as lipid ligands that stimulate various cellular responses [1,2]. In case of LPA, it induces cellular proliferation in primary ovarian carcinoma cells, prostate cancer cell lines, amnionic cells, and so on [3–5]. LPA also induces cellular migration and invasion in fibroblasts or some cancer cells, such as ovarian cancer cells [6,7]. In terms of target receptors for LPA, G-protein coupled receptor (GPCR) on plasma membrane, such as LPA_1, LPA_2, and LPA_3 have been reported [8,9]. Although LPA has been reported to regulate a

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Abbreviations: LPA, lysophosphatidic acid; GPCR, G-protein coupled receptor; LPG, lysophosphatidylglycerol; fura-2/AM, fura-2 pentaacetoxymethylester; PTX, pertussis toxin; ERK, extracellular signal regulated protein kinase; $[Ca^{2+}]_i$, intracellular calcium concentration; PLC, phospholipase C; NF-κB, nuclear factor kappa B

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plethora of cellular activity in many cell types, role of other lysophospholipids such as lysophosphatidylglycerol (LPG) on cellular (patho)physiology has not been examined.

LPG is a precursor for de novo synthesis of anionic phospholipids phosphatidylglycerol which represents ~1% of total phospholipids in most mammalian tissues and play a crucial role in the maintenance of normal function of lung, in liposome formation and endosome organization [10,11]. LPG was shown to prevent binding of LPA to a putative LPA receptor on cell-surface of mouse NIE-115 neuroblastoma cells [12]. High concentration of LPG (60 μM) also blocked intracellular calcium increase induced by LPA in HEY ovarian cancer cells [13]. However, roles of LPG in the modulation of biological responses have been poorly studied. LPG's role in various cellular activities and its mechanism of action should be investigated.

It has been demonstrated that the activation of cell surface receptors by lipid ligands such as LPA and sphingosine-1-phosphate elicits diverse intracellular signals, including $[Ca^{2+}]_i$ increase [14,15]. Calcium signaling regulates various kinds of cellular responses, including secretion and cell death [16,17]. The activation of certain types of GPCR also has been known to induce the activation of mitogen-activated protein kinase (MAPK) and Akt. Since these kinases are important mediators of intracellular signaling which result in the regulation of various crucial cellular responses, including gene expression in the nucleus, trafficking of cells, proliferation and apoptosis in various cell types [18–20]. In this study, we examined the effect of LPG on the activities of intracellular signaling in human ovarian cancer cells.

2. Materials and methods

2.1. Reagents

1-Acyl-2-hydroxy-*sn*-glycero-3-phospho-glycerol (LPG) and Na^+ lysophosphatate (LPA) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Stock solutions of the two lipids (20 mM each) were prepared by dissolving them in distilled water and subsequent sonication. Any carrier protein such as bovine serum albumin was not used for the preparation of LPG and LPA. Fura-2 pentaacetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR). 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexyl]-1H-pyrrole-2,5-dione (U-73122), 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione (U-73343), and pertussis toxin (PTX) 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, Akt antibodies, and suramin were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

OVCAR-3 human ovarian cancer cells and SK-OV3 human ovarian cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), cultured at 37 °C in a 5% CO_2 humidified incubator, and maintained in RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum (FBS). U937 (human histiocytic lymphoma cells), Jurkat (human T cell leukemia cells), MCA-102 (mouse fibroblasts), and WISH (human amniotic cells) were maintained as recommended. Human peripheral blood monocytes and neutrophils were isolated as described before [21,22]. Rat primary chondrocytes were isolated according to previous report [23].

2.3. Ca^{2+} measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using fura-2/AM [21]. Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37 °C for 50 min in fresh serum free RPMI 1640 medium with continuous stirring. 2×10^6 cells were aliquoted for each assay into Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM $CaCl_2$, and 0.2 mM EGTA). Fluorescence was measured at 500 nm at excitation wavelengths of 340 and 380 nm.

2.4. Western blot analysis

OVCAR-3 human ovarian cancer cells were plated in a 6-well plate and treated LPG for different times. The cells were then washed with cold-PBS, scraped off, and pelleted at 700 × *g* at 4 °C. The cell pellet obtained was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail), cleared by centrifugation, and the supernatant saved as a whole-cell lysate. Proteins (30 μg) were separated by 10% reducing SDS-polyacrylamide gel electrophoresis and electroblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween 20), incubated with antibodies for 4 h, washed, and re-incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase. Finally, the membrane was washed and developed using an ECL system.

2.5. Luciferase assay

Human LPA_1, LPA_2, and LPA_3 cDNAs were a gift from P.G. Suh (POSTECH, Korea). NF-κB reporter construct was purchased from Clonetech (Palo Alto, CA). HepG2 cells were plated in six-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were transfected with 2 μg of each plasmid construct for 6 h by the Lipofectamine method. After transfection, HepG2 cells were cultured in 10% FBS containing DMEM medium with LPA (10 μM) or LPG (2 μM) for 20 h. Cells were lysed with lysis buffer (20 mM Tris–HCl, pH 7.8, 1% Triton X-100, 150 mM NaCl, 2 mM DTT). The cell lysate 30 μl was mixed with luciferase activity assay reagent 30 μl and luminescence produced for 15 s was measured using luminoskan (labsystems).

2.6. Statistics

The results are expressed as means ± S.E. of the number of determinations indicated. Statistical significance of differ-

ences was determined by ANOVA. Significance was accepted when $P < 0.05$.

3. Results

3.1.1. LPG stimulates calcium mobilization in OVCAR-3 ovarian cancer cells

We examined the effect of LPG upon $[Ca^{2+}]_i$ increase in OVCAR-3 ovarian cancer cells. As shown in Fig. 1A, the stimulation of OVCAR-3 ovarian cancer cells with 2 μM of LPG caused a $[Ca^{2+}]_i$ increase in the presence or in the absence of extracellular calcium. Concentration-dependency of LPG-induced $[Ca^{2+}]_i$ increase was also investigated in OVCAR-3 ovarian cancer cells. Increase of $[Ca^{2+}]_i$ was apparent at 100 nM of LPG and maximal activity was observed at 1–2 μM (Fig. 1B).

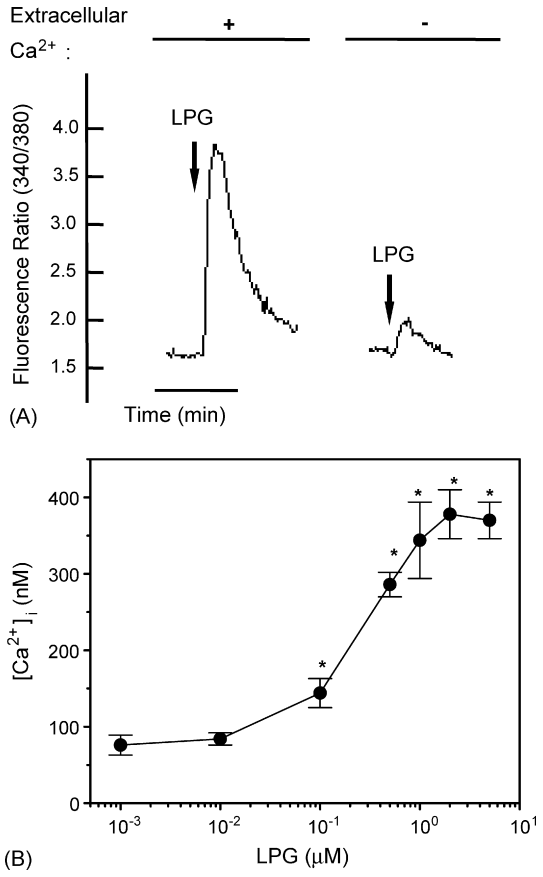

Fig. 1 – Effect of LPG on $[Ca^{2+}]_i$ increase in OVCAR-3 human ovarian cancer cells. OVCAR-3 human ovarian cancer cells were stimulated with 2 μM of LPG in the presence or absence of extracellular calcium (2 mM), and $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM. Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of five independent experiments (A). OVCAR-3 human ovarian cancer cells were stimulated by various concentrations of LPG in the presence of extracellular calcium (2 mM). The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as mean ± S.E. of three independent experiments, which performed in duplicate (B). *Results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (vehicle treated).

3.2. LPG-induced $[Ca^{2+}]_i$ increase is mediated via G-proteins and PLC

To determine the role of phospholipase C (PLC) on LPG-induced $[Ca^{2+}]_i$ increase, we pretreated the cells with a specific PLC inhibitor, U-73122 or with its inactive analogue U-73343. Fig. 2A shows that U-73122, but not U-73343, completely inhibited LPG-induced $[Ca^{2+}]_i$ increase. This result indicates that LPG stimulates $[Ca^{2+}]_i$ increase via PLC activation in OVCAR-3 ovarian cancer cells. We also examined the effect of PTX, a specific inhibitor of $G_{i/o}$ type G-proteins, on LPG-induced $[Ca^{2+}]_i$ increase. When OVCAR-3 ovarian cancer cells were preincubated with 100 ng/ml of PTX prior to being stimulated with 2 μM LPG, LPG-induced $[Ca^{2+}]_i$ increase was almost completely inhibited (Fig. 2B). These results indicate that LPG stimulates $[Ca^{2+}]_i$ increase via PTX-sensitive G-proteins. As a negative control, we observed that ATP also stimulated $[Ca^{2+}]_i$ increase in OVCAR-3, and ATP-stimulated $[Ca^{2+}]_i$ increase was not affected by PTX treatment (data not shown), indicating specificity of PTX-sensitive G-proteins for LPG-induced $[Ca^{2+}]_i$ increase.

3.3. Cell type specificity of LPG

The fact that LPG stimulates OVCAR-3 cells led us to look for effects of LPG on other cell types. We examined the effects of LPG on intracellular calcium release in multiple cell types. At first we found that LPG stimulated $[Ca^{2+}]_i$ rise in two human ovarian cancer cells, OVCAR-3 and SK-OV3 (Fig. 3). U937 human promonocytic cells, Jurkat T cell leukemia cells, MCA-102 mouse fibrosarcoma cells, WISH human amniotic cells, rat primary chondrocytes, human primary monocytes and human primary neutrophils showed no response to LPG in terms of $[Ca^{2+}]_i$ rise (Fig. 3). The results suggest that the effect of LPG is specific for human ovarian cancer cells.

3.4. LPG stimulates ERK in OVCAR-3 ovarian cancer cells

ERK has been reported to mediate extracellular signals to the nucleus in various cell types [24]. In this study, we examined whether LPG stimulates ERK by Western blotting with anti-phospho-ERK antibody. When OVCAR-3 cells were stimulated with 2 μM LPG for different times, the phosphorylation level of ERK transiently increased, showing maximal activity after 5–10 min of stimulation (Fig. 4A). We also examined the effect of PTX on LPG-induced ERK phosphorylation. As shown in Fig. 4A, LPG-induced ERK phosphorylation was not affected by preincubation of OVCAR-3 ovarian cancer cells with 100 ng/ml of PTX prior to LPG stimulation. The result indicates that LPG stimulates ERK activity independently of PTX-sensitive G-proteins. Moreover, we found that stimulation of OVCAR-3 ovarian cancer cells with various concentrations of LPG induced ERK phosphorylation. LPG induced ERK phosphorylation in a concentration-dependent manner, showing maximal activity at 1–2 μM (Fig. 4B).

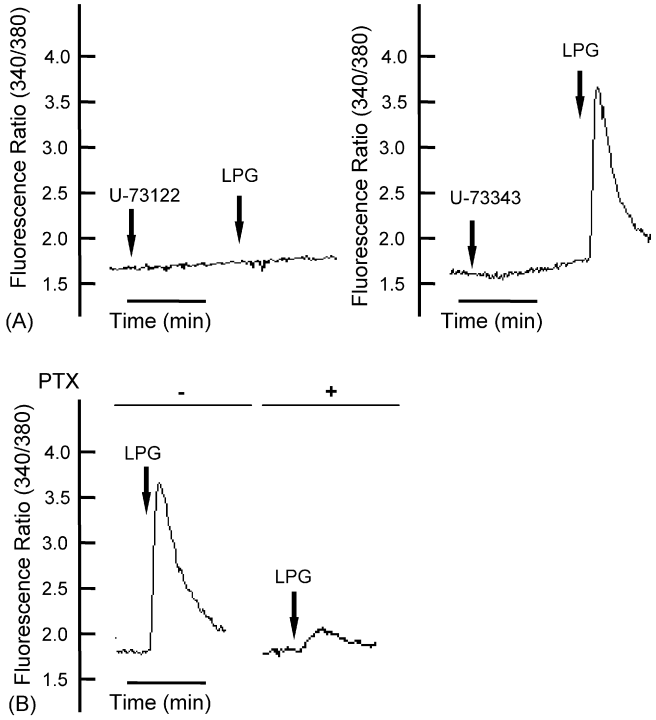

Fig. 2 – LPG-induced Ca^{2+} signaling is PTX-sensitive in OVCAR-3 cells. OVCAR-3 cells were pretreated with 5 μM of U-73122 or 5 μM of U-73343 prior to 2 μM of LPG, and $[Ca^{2+}]_i$ was determined (A). OVCAR-3 cells were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. OVCAR-3 cells were then loaded with fura-2/AM and $[Ca^{2+}]_i$ was determined fluorometrically after stimulation with 2 μM of LPG (B). Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of four independent experiments (A and B).

3.5. *LPG stimulates Akt activity in OVCAR-3 ovarian cancer cells*

Akt has been reported to play important roles in the regulation of several cellular responses, such as cell migration and cell survival [25]. Here, we used Western blot analysis with specific antibody against phospho-Akt to determine whether LPG stimulates Akt activity. When OVCAR-3 cells were stimulated with 2 μM LPG for different times, Akt phosphorylation was transiently increased, showing maximal activity after 5 min of stimulation and sustained for 30 min after stimulation (Fig. 5A). The effect of PTX on LPG-induced Akt activity was also examined. LPG-induced Akt phosphorylation was not affected by PTX treatment (Fig. 5A). It suggests that LPG-stimulated Akt activation is independent of PTX-sensitive G-proteins. In addition, we also examined the concentration-dependency of LPG-induced Akt activation. When OVCAR-3 cells were stimulated with different concentrations of LPG, Akt was activated in a concentration-dependent manner (Fig. 5B). At 100 nM LPG caused significant Akt activation and maximal activation was observed at 2 μM (Fig. 5B).

3.6. *LPG-induced $[Ca^{2+}]_i$ increase is independent of LPA_1, LPA_2, and LPA_3*

It has been known that suramin is an inhibitor of GPCR signaling and it has been used for the study of the involvement of GPCR on some agonists-induced signaling [26]. Here, we investigated the effect of suramin on LPG-induced $[Ca^{2+}]_i$ rise in OVCAR-3 cells. As shown in Fig. 6A, suramin (50 μM) almost completely blocked LPG-induced $[Ca^{2+}]_i$ increase in OVCAR-3 cells. The result indicates that LPG stimulates certain GPCR(s) on OVCAR-3 cells.

LPA is a well-known lysophospholipid that stimulates $[Ca^{2+}]_i$ rise in human ovarian cancer cells [13]. Thus, we suspected a possibility of that LPG utilizes GPCRs for LPA to elicit Ca^{2+} response, and conducted desensitization experiments. As shown in Fig. 6B, stimulation of OVCAR-3 ovarian cancer cells with LPG desensitized cells, resulting in no response to the second LPG stimulation, meaning homologous desensitization. The homologous desensitization was also observed with LPA. However, LPG-desensitized OVCAR-3 ovarian cancer cells responded to LPA (Fig. 6B). In the reverse cases, LPA-desensitized OVCAR-3 ovarian cancer cells were not responding to LPG, meaning heterologous desensitization. Therefore, these results suggest that LPG may share the same receptor(s) with LPA or that the downstream signaling pathways converge or interact with those of LPA.

To further examine whether LPG share the same receptors with LPA, we utilized nuclear factor kappaB (NF-κB)-driven luciferase activity. At first we observed that treatment of LPA or LPG induced NF-κB-driven luciferase activity in NF-κB-luciferase transfected OVCAR-3 cells (data not shown). And then, we investigated the effect of LPA or LPG on NF-κB activity in LPA_1, LPA_2, or LPA_3-transfected HepG2 cells. As shown in Fig. 6C, treatment of LPA elicited NF-κB-driven luciferase

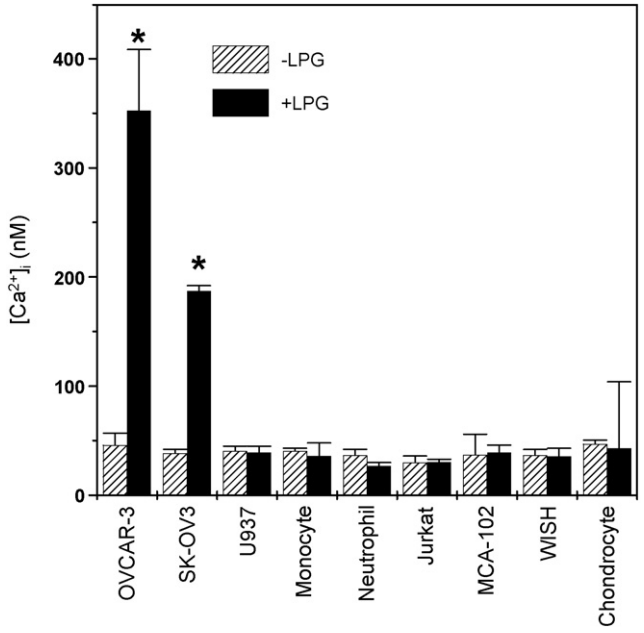

Fig. 3 – Cell type specificity of LPG action. OVCAR-3, SK-OV3, U937, Jurkat, human primary monocytes, neutrophils, MCA-102, WISH, and rat primary chondrocytes were loaded with fura-2 and stimulated with effective concentrations of LPG (2 μM). The peak level of the $[Ca^{2+}]_i$ increase was recorded. Data are presented are mean ± S.E. from at least three independent experiments. *Results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (vehicle treated).

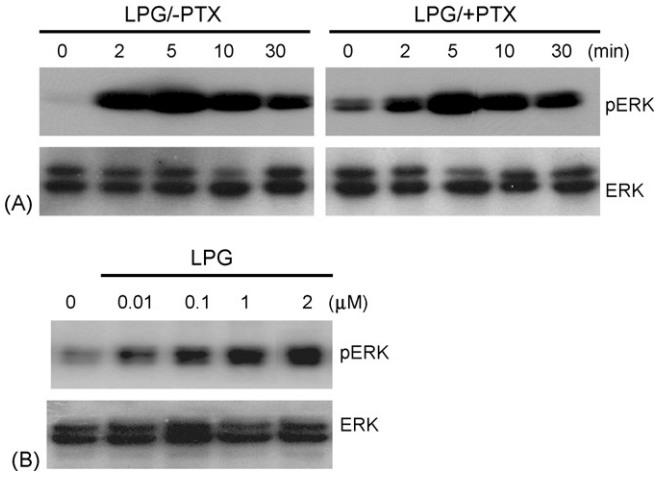

Fig. 4 – Activation of ERK by LPG in OVCAR-3 ovarian cancer cells. OVCAR-3 cells, preincubated in the absence (left panel) or presence (right panel) of 100 ng/ml of PTX for 24 h, were stimulated with 2 μM of LPG for various times (A). OVCAR-3 cells were stimulated with various concentrations of LPG for 5 min (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phospho-ERK (pERK) was determined by immunoblotting using anti-phospho-ERK antibody. The results shown are representative of at least three independent experiments (A and B).

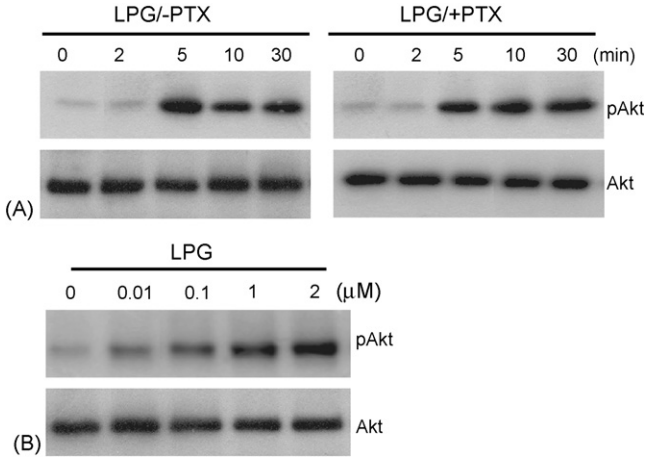

Fig. 5 – Activation of Akt by LPG in OVCAR-3 ovarian cancer cells. OVCAR-3 cells, preincubated in the absence (left panel) or presence (right panel) of 100 ng/ml of PTX for 24 h, were stimulated with 2 μM of LPG for various times (A). OVCAR-3 cells were stimulated with various concentrations of LPG for 5 min (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phospho-Akt (pAkt) was determined by immunoblotting using anti-phospho-Akt antibody. The results shown are representative of at least three independent experiments (A and B).

activity in LPA_1, LPA_2, or LPA_3-transfected HepG2 cells. However, LPG failed to stimulate NF-κB-driven luciferase activity in the cells (Fig. 6C). These findings strongly indicate that LPG is not an agonist for the three known LPA receptors; LPA_1, LPA_2 and LPA_3.

4. Discussion

Previous reports demonstrated that lysophospholipids, including LPA and lysophosphatidylcholine have cytokine-like properties [1–5]. LPA activates ovarian cancer cell lines, resulting in $[Ca^{2+}]_i$ increase and proliferation [13]. LPA also acts as a survival signal for ovarian cancer cells against chemotherapeutic agents [27]. Lysophosphatidylcholine also acts as a lipid ligand that stimulates oxidant production in neutrophils, resulting in therapeutic effect against experimental sepsis [28,29]. Very recently, we and others demonstrated that lysophosphatidylserine also stimulates intracellular calcium increase and degranulation activity via cell surface-bound GPCR in several cells, such as leukemic cells, fibroblasts, and mast cells [30–32]. In this study, we found that LPG also act as a lipid ligand and stimulates intracellular Ca^{2+} increase, activation of ERK and Akt in human ovarian cancer cells. Although LPG receptor has not been identified until now, a certain cell surface receptor, which couples to PTX-sensitive G-proteins, may be activated by LPG.

In this study, we found that LPG induced increase of intracellular Ca^{2+} concentration in a unique way; the LPG-induced Ca^{2+} response was completely inhibited by suramin treatment, suggesting the involvement of GPCR (Fig. 6A). LPG-

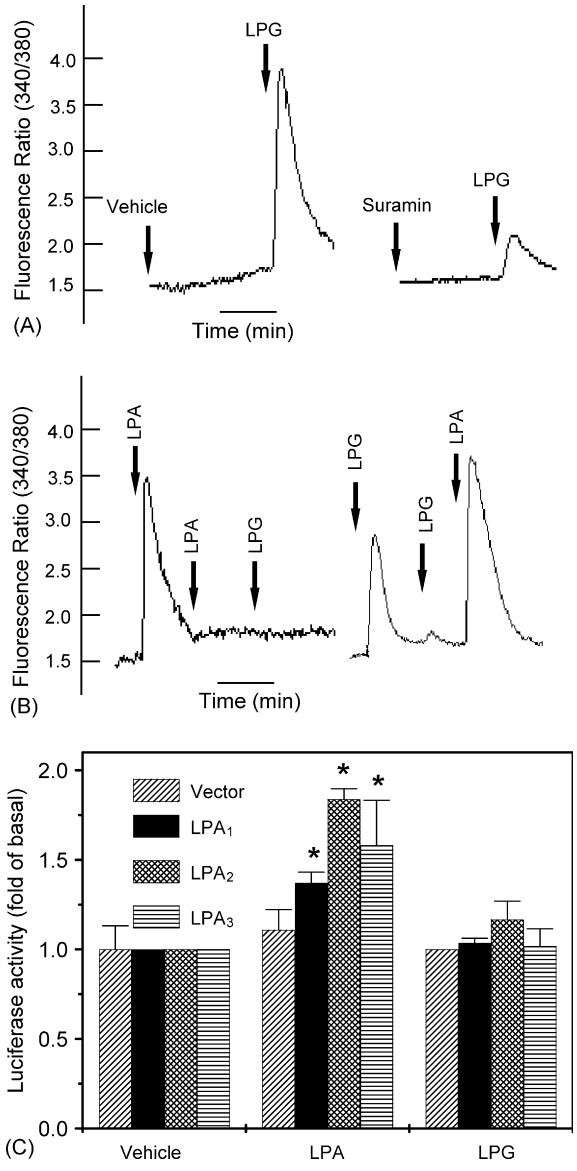

Fig. 6 – LPG-induced $[Ca^{2+}]_i$ increase is independent of LPA receptors in OVCAR-3 cells. Fura-2/AM loaded OVCAR-3 cells were pretreated with vehicle (DW) or 50 μM of suramin prior to 2 μM of LPG (A). Fura-2/AM loaded OVCAR-3 cells were challenged with 2 μM LPG or 10 μM LPA at the time indicated by the arrow (B). Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of four independent experiments (A and B). LPA_1, LPA_2, or LPA_3 cDNA and NF-κB-luciferase cDNA were co-transfected into HepG2 cells. Cells were stimulated with LPA (10 μM) or LPG (2 μM) for 20 h (C). Luciferase activities were measured in cell lysates using luciferase reporter gene assays. The data shown represent mean ± S.E. from three separate experiments performed in duplicate (C). *Results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (vehicle treated).

induced Ca^{2+} response was also desensitized by prior treatment of LPG and LPA, however, LPA-induced Ca^{2+} response was not desensitized by prior treatment of LPG (Fig. 6B). To examine whether LPG acts on LPA receptors, we investigated the effect of LPG on LPA receptor-mediated signaling. We demonstrated that LPG failed to stimulate NF-κB-driven luciferase activity in HepG2 cells expressing LPA_1, LPA_2 or LPA_3 exogenously (Fig. 6C). A novel GPCR, GPR23, which is structurally distinct from the known LPA receptors (LPA_1, LPA_2 or LPA_3), has been reported as an LPA receptor [33]. Furthermore high expression of GPR23 mRNA was observed in ovary [33]. However, LPG did not compete LPA binding to GPR23, suggesting LPG does not share GPR23 [33]. Taken together the results indicate that LPG has its own receptor, which is distinct from that of LPA, in OVCAR-3 ovarian cancer cells.

We also examined the possibility that LPG and LPA receptors share a common downstream mechanism. As shown in Fig. 6B, while LPA induced heterologous desensitization to a subsequent challenge of LPG, LPG failed to heterologously desensitize the LPA signaling. The heterologous desensitization by LPA to subsequent LPG might be induced by LPA sharing a downstream desensitization mechanism of LPG. And none of heterologous desensitization by LPG to subsequent LPA may imply that the shared downstream desensitization mechanism of LPG was not enough to desensitize the subsequent LPA response. Although LPG was shown not to stimulate LPA_1, LPA_2, or LPA_3 (Fig. 6C), currently we are not able to exclude the possibility of that LPA acts as an agonist on the putative LPG receptor(s). This possibility could explain why LPA stimulation desensitizes subsequent LPG response, but not the reverse.

In our study, we investigated the effect of PTX, which specifically inactivates G_i/G_o-mediated signaling pathways, on LPG-induced signaling. When OVCAR-3 ovarian cancer cells were treated with 100 ng/ml of PTX for 24 h prior to LPG stimulation, LPG-induced $[Ca^{2+}]_i$ increase was almost completely inhibited (Fig. 2B). This result implies that LPG utilizes PTX-sensitive GPCR, resulting in PLC activation and $[Ca^{2+}]_i$ increase. However, when OVCAR-3 ovarian cancer cells were pretreated with 100 ng/ml of PTX for 24 h prior to LPG stimulation, LPG-induced ERK and Akt phosphorylation were not inhibited (Figs. 4A and 5A). These results suggest that LPG modulates activities of ERK and Akt independently of PTX-sensitive G-proteins in OVCAR-3 cells. Taken together, the results suggest that LPG induces at least two different signaling cascades; i.e., PTX-sensitive G-protein-dependent $[Ca^{2+}]_i$ increase and PTX-sensitive G-protein-independent ERK and Akt activation in OVCAR-3 human ovarian cancer cells. To our knowledge, it will be the first report that demonstrates the role of PTX-sensitive trimeric G-proteins or GPCRs for LPG. Since this study is the only report upon the role of LPG on calcium signaling in human ovarian cancer cells, further studies on the pathological roles of LPG and on LPG specific cell surface receptor(s) in human ovarian cancer cells are required.

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