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Stimulation of early gene induction and cell proliferation by lysophosphatidic acid in human amnion-derived WISH cells: role of phospholipase D-mediated pathway

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Abstract

Human-amniotic WISH cells express the lysophosphatidic acid (LPA) receptor, LPA_1, LPA_2 but not LPA_3. When WISH cells were stimulated with LPA, phospholipase D (PLD) activation was dramatically induced via a cytosolic calcium increase and protein kinase C activation. We also found that LPA stimulated two kinds of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p38 kinase via PLD-dependent signaling pathways in WISH cells. In terms of the LPA-mediated functional modulation of WISH cells, we observed that LPA stimulates the induction of two early genes (c-Jun and c-Fos) and cellular proliferation in WISH cells. We examined the signaling pathways involved in LPA-mediated cellular responses. LPA-induced early gene induction was completely blocked by normal butanol (*n*-butanol) but not by *t*-butanol, suggesting that PLD activity is essentially required for the process. PD98059 (2′-amino-3′-methoxyflavone) but not SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) also significantly blocked LPA-induced early gene induction, suggesting a crucial role for ERK. Pertussis toxin (PTX) did not affect on the LPA-induced early gene induction and ERK activation, ruling out the role of Gi/o protein(s) in the process. The cellular proliferation of WISH cells was also dramatically inhibited by *n*-butanol or PD98059. This study demonstrates the physiological role of LPA on the modulation of early gene induction and on WISH cell proliferation, and the crucial role played by PLD in the process.

Keywords: Amniotic cells; LPA; PLD; Early gene; Proliferation; ERK

1. Introduction

Lysophosphatidic acid (LPA) is an important lipid mediator, with wide-ranging physiological activities [1,2]. LPA has been reported to induce cellular proliferation in several cell types, including primary ovarian carcinoma cells and prostate cancer cell lines [3,4]. Some reports have demonstrated that LPA dramatically affects the actin cytoskeleton, especially in neuronal origin cells [5]. LPA also regulates the tissue remodeling processes occurring during growth, differentiation, and the regression of the corpus luteum [6]. In terms of LPA cell surface receptors, a family of G-protein coupled receptors called endothelial differentiation genes (EDGs) has been shown to contain specific LPA receptors. This family includes EDG2/LPA_1, EDG4/LPA_2, and EDG7/LPA_3 [7,8]. Although many previous reports have demonstrated the pivotal role of LPA in the modulation of several biological responses, the expression of LPA receptors in amnion cells and the role of LPA in these cells have not been previously studied.

LPA can be produced in activated cells by the hydrolysis of preexisting cellular membranes phospholipids [9]. Serum is regarded as a major reservoir of LPA, and serum LPA can be generated by platelets activated by thrombin

Abbreviations: LPA, lysophosphatidic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; Fura-2/AM, fura-2 pentaacetoxymethylester; U73122, 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; PLD, phospholipase D; PBt, phosphatidylbutanol; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated protein kinase; PD98059, 2′-Amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; PTX, pertussis toxin

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[10]. Recently, reproductive tissues have been reported to be important source of locally synthesized LPA [11]. Since LPA is present in follicular fluid and in the ascites of ovarian cancer patients, LPA in the ovary is considered to be produced by the ovary itself [12]. However, the role of LPA in the regulation of reproduction has not been fully investigated.

In this study, we aimed to investigate whether LPA receptors are expressed on human amnion-derived WISH cells and LPA modulate cellular activity of the cells. We also investigated the signaling pathways involved in the LPA-mediated WISH cell activation.

2. Materials and methods

2.1. Reagents

The reverse transcription-polymerase chain reaction kit was purchased from Invitrogen Corporation (Carlsbad, CA), and fetal calf serum from Hyclone (Logan, UT). Enhanced chemiluminescence reagents were from Amersham Biosciences (Piscataway, NJ), and phospho-ERK1/2, phospho-p38 kinase, ERK2, c-Jun, and c-Fos antibodies from New England Biolabs (Beverly, MA). PD98059 (2′-amino-3′-methoxyflavone) and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were obtained from Calbiochem (San Diego, CA) and were dissolved in dimethyl sulfoxide before being added to cell culture. The final concentrations of dimethyl sulfoxide in culture were 0.1% or less.

2.2. Cell culture

The WISH human amnion cell line was obtained from the American Type Culture Collection (Manassas, VA). WISH cells were cultured in RPMI 1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. The cells were subcultured twice weekly by trypsinization and seeded in either 12- (2×10^5 cells per well) or 6-well plates (5×10^5 cells per well).

2.3. RT-PCR analysis

mRNA was isolated using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV reverse transcriptase, and pd(N)6 primers (GIBCO BRL, Gaithersburg, MD) were used to obtain cDNA. The primers used for the RT-PCR analysis have been reported previously [13]. The sequences of the primer used were as follows; LPA_1 receptor (349 bp product): sense, 5′-TCT TCT GGG CCA TTT TCA AC-3′; anti-sense, 5′-TGC CTR AAG GTG GCG CTC AT-3′. LPA_2 receptor (798 bp product): sense, 5′-CCT ACC TCT TCC TCA TGT TC-3′; anti-sense, 5′-TAA AGG GTG GAG TCC ATC AG-3′. LPA_3 receptor (382 bp product): sense, 5′-GGA ATT GCC TCT GCA ACA TCT-3′; anti-sense, 5′-GAG TAG ATG ATG GGG TTC A-3′. GAPDH (246 bp product): sense, 5′-GATGACATCAAGAAGGTGGTGAA-3′, anti-sense, 5′-GTC TTA CTC CTT GGA GGC CAT GT-3′. We ran 30 PCR cycles of 94 °C (denaturation, 1 min), 62 °C (annealing, 1 min), and 72 °C (extension, 1 min). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

2.4. $[Ca^{2+}]_i$ measurements

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined using Grynkiewicz's method using fura-2 pentaacetoxymethylester (fura-2/AM) [14]. 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 Ca^{2+} free Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, and 0.2 mM EGTA). Fluorescence was measured at 500 nm at excitation wavelengths of 340 and 380 nm, and the calculated fluorescence ratio was translated into $[Ca^{2+}]_i$.

2.5. Measurement of phosphatidylbutanol formation

The production of phosphatidylbutanol (PBt) was determined using a slight modification of the method previously reported [15]. WISH cells were seeded at 1×10^6 cells/ml in RPMI 1640 medium containing 10% FBS, and cultured for 20 h with serum free RPMI1640 medium. The cells were then loaded with [^{3}H]myristic acid (5 μCi/ml) for 4 h at 37 °C, washed twice with serum-free RPMI 1640 medium, and stimulated with LPA in the presence of 0.5% normal butanol (*n*-butanol). After 30 min, the reactions were quenched by adding 0.5 ml ice-cold methanol, and the medium was aspirated. Total lipids were extracted after adding 0.5 ml of chloroform and 0.5 ml of 1 M NaCl, by vigorous vortexing. The lower phase obtained after centrifugation at $550 \times g$ for 10 min was dried under nitrogen. The lipids were then dissolved in chloroform: methanol (95:5), spotted onto silica gel 60 TLC plates, and separated using a solvent system containing chloroform:methanol:acetic acid (90:10:10), as described previously [15]. In order to determine the quantity of PBt and the total lipids, we used a Fuji BAS-2000 image analyzer (Fuji Film Co., Ltd.).

2.6. Western blot analysis

WISH cells were plated in a six-well plate and treated LPA for different times. The cells were then washed with cold-PBS, scraped off, and pelleted at $700 \times g$ 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, 0.5% Nonidet P-40, 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 enhanced ECL system.

2.7. Cellular proliferation assay

WISH cells were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2. For LPA treatment, the cells were seeded in 24-well plates, in triplicate, at 5×10^4 cells per well. Complete medium was replaced with serum free medium to starve the cells for 24 h. LPA was added to the cultures to promote growth activity with or without several kinase inhibitors. [^{3}H]thymidine (1 μCi/ml) was added and incubation was continued for 24 h. The medium was then removed and the cells were fixed with 5% cold trichloroacetic acid (TCA) for 15 min. TCA precipitated material was solubilized in 1 M NaOH for 1 h and neutralized by adding 1 M HCl. [^{3}H]thymidine uptake was determined by liquid scintillation counting.

2.8. Statistics

The results are expressed as means ± S.E. of the number of determinations indicated. The student's *t*-test was used to compare individual treatments with their respective control values. Significance was accepted when $P < 0.05$.

3. Results

3.1. Expression of LPA receptors in WISH cells

Previously we reported that human amnion-derived WISH cells express cell surface receptors for S1P [16]. In this study, we investigated whether cell surface receptors for LPA are expressed on WISH cells. To determine which LPA receptor isoforms are expressed on WISH cells, we analyzed the mRNA expressions of different LPA receptors by semi-quantitative RT-PCR. As shown in Fig. 1, WISH cells expressed two forms of LPA receptor, namely, LPA_1 and LPA_2 (Fig. 1); we were unable to detect LPA_3 expression (Fig. 1). We confirmed that the RT-PCR product obtained without the addition of reverse transcriptase did not contain a DNA band (Fig. 1), indicating that WISH cells express LPA_1 and LPA_2.

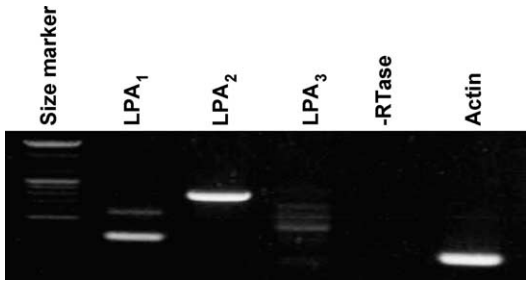

Fig. 1. Expression of LPA receptors in WISH cells. RT-PCR analysis was performed on mRNA isolated from cultured WISH cells. The data presented are representative of three independent experiments. RTase indicates M-MLV reverse transcriptase.

3.2. Stimulation of intracellular calcium increase via phospholipase C activation by LPA in WISH cells

Several previous reports have demonstrated that the activation of LPA-specific receptors by LPA binding causes diverse intracellular signals, including intracellular calcium increases [3–5]. To confirm that LPA receptors on WISH cells are functional, we examined the effect of LPA upon intracellular calcium concentration ($[Ca^{2+}]_i$) in WISH cells. As shown in Fig. 2A, the stimulation of WISH cells with 5 μM of LPA caused a $[Ca^{2+}]_i$ increase in the presence or in the absence of extracellular calcium. One of the well-known mechanism of $[Ca^{2+}]_i$ increase in the absence of extracellular calcium is the phospholipase C (PLC)-dependent inositol-1,4,5-trisphosphates-mediated response [17]. To determine the role of

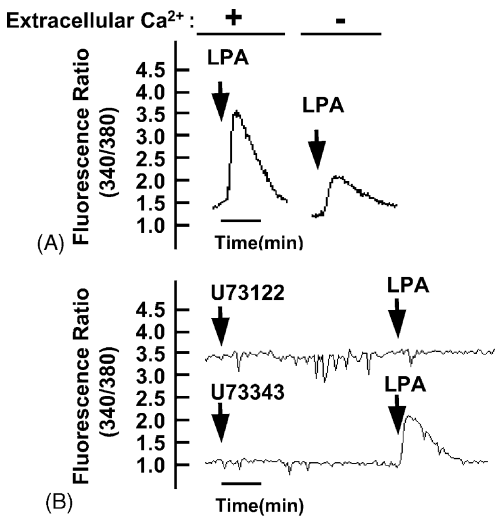

Fig. 2. The effect of LPA on intracellular calcium increase in WISH cells. WISH cells were stimulated with 5 μM of LPA, and $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM. Peak $[Ca^{2+}]_i$ levels were recorded (A). WISH cells were pretreated with 5 μM of U73122 or 5 μM of U73343 prior to 5 μM of LPA, and $[Ca^{2+}]_i$ was determined (B). Data are representative of four independent experiments (A, B).

PLC on LPA-induced $[Ca^{2+}]_i$ increase, we pretreated the cells with a specific PLC inhibitor, U-73122 or with its inactive analogue U-73343. Fig. 2B shows that U-73122, but not U-73343, completely inhibited LPA-induced $[Ca^{2+}]_i$ increase. This result indicates that LPA stimulates $[Ca^{2+}]_i$ increase via PLC activation in WISH cells.

3.3. Activation of phospholipase D via protein kinase C- and Ca^{2+}-dependent signaling by LPA in WISH cells

Phospholipase D (PLD) is regarded as a key enzyme, which is involved in the modulation of several functions in several cell types [18,19]. In this study, we examined the effect of LPA on PLD activity in WISH cells by using the transphosphatidylation reaction, which is characteristic of PLD in the presence of 0.5% *n*-butanol. LPA stimulated the formation of PBt in a concentration dependent manner, with maximal activity at 5 μM (Fig. 3A). The kinetics of LPA-stimulated PBt formation was also examined. When WISH cells were stimulated with 5 μM LPA for various times, PBt formation dramatically increased within 2 min of stimulation and plateaued at 5 min (Fig. 3B).

In order to investigate the signaling pathway leading to PLD activation by LPA, we examined the roles of several signaling molecules. The addition of an effective concentration (5 μM) of the PLC-inhibitor (1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; U73122) or the PKC-inhibitor (GF109203X) almost completely inhibited LPA-induced PLD activation (Fig. 3C), which was also almost completely inhibited when WISH cells were preincubated with 10 μM BAPTA/AM prior to LPA stimulation (Fig. 3C). However, pretreatment with LY294002, a phosphatidylinositol 3-kinase-selective inhibitor, did not affect the PLD activity induced by LPA (Fig. 3C), suggesting that LPA stimulates PLD activation in a PLC/Ca^{2+}- and PKC-dependent manner in WISH cells.

3.4. Role of PLD on the stimulation of extracellular signal-regulated protein kinase phosphorylation by LPA in WISH cells

Mitogen-activated protein kinase (MAPK) has been reported to mediate extracellular signals to the nucleus in various cell types [20]. In this study, we examined whether LPA stimulates MAPKs by Western blotting with anti-phospho-specific antibodies to each enzyme. When WISH cells were stimulated with 5 μM LPA for different times, the phosphorylation level of extracellular signal regulated protein kinase (ERK) transiently increased, showing maximal activity after 2–5 min of stimulation (Fig. 4A), and returning to the baseline 10 min after stimulation (Fig. 4A). Another important MAPK, p38 kinase, was also transiently phosphorylated by LPA stimulation with kinetics that resembled those

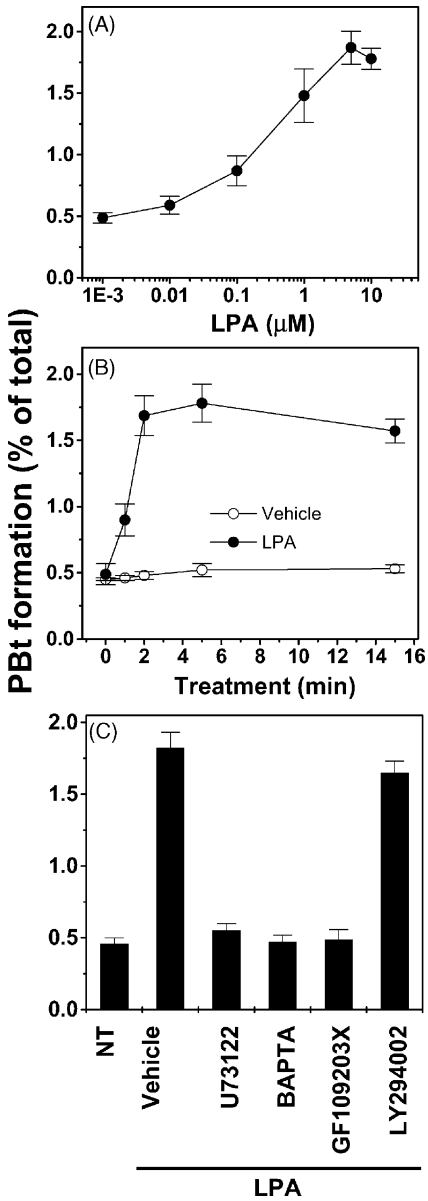

Fig. 3. Effect of LPA on the formation of PBt in WISH cells. WISH cells were cultured with serum free medium for 20 h, and then labeled with [^{3}H]myristic acid (2 μCi/ml) for 4 h. After three washes with PBS the labeled cells were stimulated with various concentrations of LPA in the presence of 0.5% *n*-butanol for 15 min (A) or with 5 μM of LPA in the presence of 0.5% *n*-butanol for various times (B). [^{3}H]myristic acid-labeled WISH cells were pretreated with U73122 (5 μM), BAPTA/AM (10 μM), GF109203X (5 μM), or LY294002 (50 μM), or with vehicle alone (C) for 15 min, and then stimulated with LPA (5 μM) for 15 min in the presence of 0.5% *n*-butanol. The amount of [^{3}H]PBt formed is expressed as a percentage of total labeled lipids. Data are average values ± S.E. of three independent experiments performed in triplicate.

of ERK phosphorylation (Fig. 4A). Many studies have demonstrated that *n*-butanol exerts its anti-PLD effect in part by suppressing PA formation [21–23]. Therefore, we examined whether *n*-butanol inhibits LPA-induced ERK phosphorylation in WISH cells, by stimulating WHSH cells with LPA in the presence of 0.5% *n*-butanol for 5 min. As shown in Fig. 4B, *n*-butanol inhibited

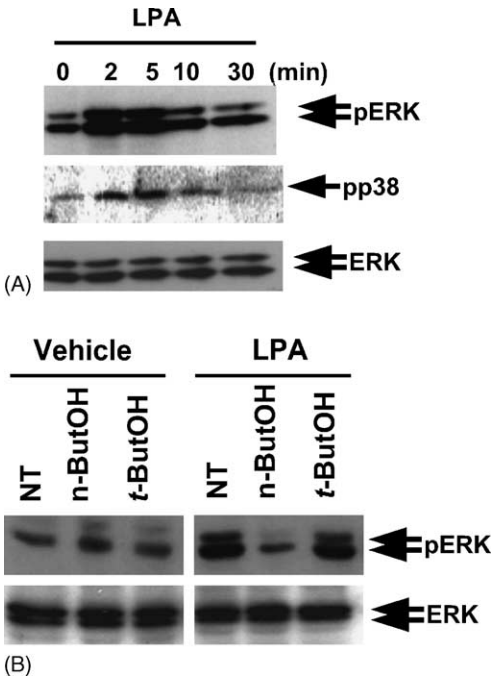

Fig. 4. Activation of MAPKs by LPA in WISH cells. WISH cells were stimulated with 5 μM of LPA for various times (A). The cells were then stimulated with vehicle or 5 μM LPA in the presence of 0.5% *n*-butanol or 0.5% *t*-butanol (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK or p38 kinase was determined by immunoblotting using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody. The results shown are representative of at least three independent experiments.

LPA-induced ERK phosphorylation, but *t*-butanol did not (Fig. 4B). These results indicate that LPA stimulates ERK phosphorylation via a PLD-mediated pathway.

3.5. Early gene induction by LPA in WISH cells

In order to examine the effect of LPA on the regulation of gene expression, we investigated the effect of LPA on early gene induction. As shown in Fig. 5A, the stimulation of WISH cells with 5 μM of LPA enhanced the expression of c-Fos at 1–3 h later (Fig. 5A). The stimulation of WISH cells with 5 μM of LPA also enhanced c-Jun expression, which showed maximal activity in 1–6 h after treatment (Fig. 5A). We investigated the role of the two MAPKs (ERK and p38 kinase) on LPA-induced early gene induction using two different MAPK inhibitors. PD98059, a selective MEK inhibitor, was found to block LPA-induced early gene induction (Fig. 5B). However, SB203580, a selective p38 kinase inhibitor, did not affect LPA-induced early gene induction. These results indicate that MEK-dependent ERK activity, but not p38 kinase activity, is essentially involved in LPA-induced early gene induction. We also examined the role of PLD in LPA-induced early gene induction in WISH cells. As shown in Fig. 5C, LPA-induced early gene induction was inhibited by 0.5% *n*-butanol but not by 0.5% *t*-butanol, suggesting the crucial role played by PLD in this process.

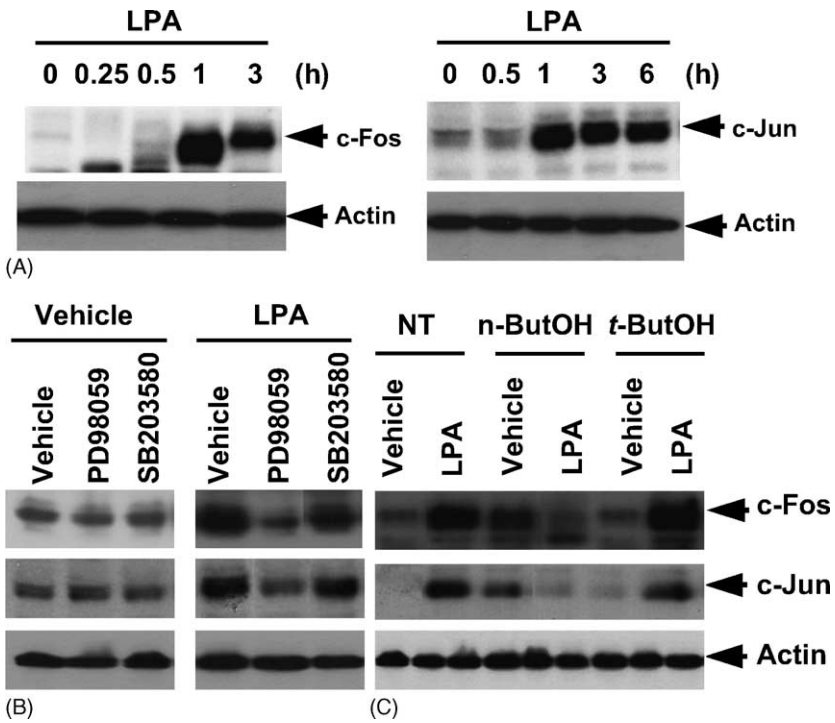

Fig. 5. Effect of LPA on early gene induction in WISH cells. WISH cells were treated with 5 μM of LPA for various times (A); or cells were preincubated with PD98059 (50 μM) or SB203580 (20 μM) for 15 min prior to adding 5 μM LPA for 1 h (B). Cells were stimulated with vehicle or 5 μM LPA in the presence of 0.5% *n*-butanol or 0.5% *t*-butanol (C). LPA-treated WISH cells were lysed and the extracted protein was immunodetected with c-Fos or c-Jun specific antibodies. Western blotting analysis was performed with anti-actin antibody to confirm equal protein loadings. The results shown are representative of at least three independent experiments.

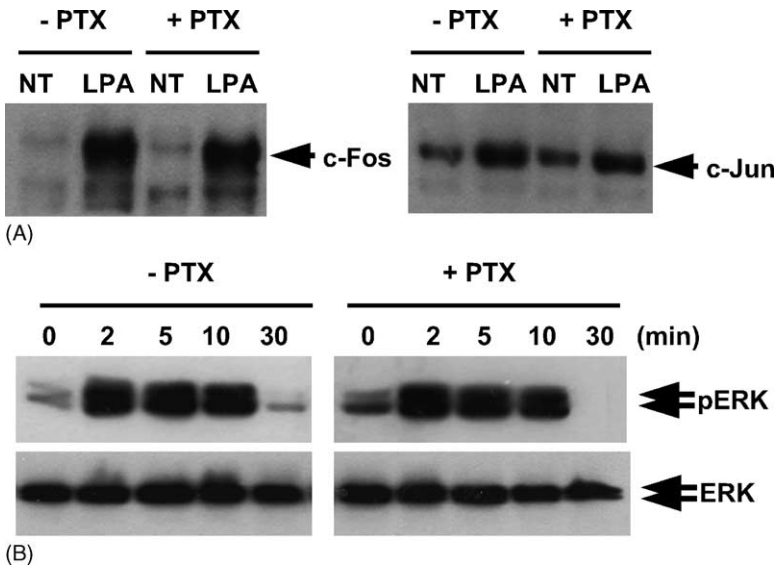

Fig. 6. PTX does not block LPA-induced signaling in WISH cells. WISH cells were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were stimulated with 5 μM of LPA for 1 h (A). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and the expression levels of c-Jun and c-Fos were determined by immunoblot analysis using specific antibodies (A). Cells were stimulated with 5 μM of LPA for 5 min (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK was determined by immunoblotting using anti-phospho-ERK antibody (B). The results shown are representative of at least three independent experiments (A, B).

3.6. Involvement of pertussis toxin (PTX)-insensitive pathway on the LPA-induced early gene induction

We investigated the role of PTX-sensitive G-protein on LPA-induced early gene induction. Cultured WISH cells were preincubated with 100 ng/ml of PTX prior to being stimulated with 5 μM LPA. We found that pretreatment with PTX did not block early gene induction by LPA (Fig. 6A), showing that LPA induces early gene induction in a PTX-insensitive manner. Fig. 5B shows that LPA-stimulated early gene induction was inhibited by >95% by PD98059, thus indicating the critical role played by ERK in this process. We also examined the effect of PTX on LPA-induced ERK phosphorylation. When WISH cells were preincubated with 100 ng/ml of PTX prior to being stimulated with 5 μM LPA, LPA-induced ERK phosphorylation was not found to be blocked (Fig. 6B). This result supports our notion that LPA stimulates early gene induction via a PTX-insensitive pathway and that ERK is critically involved in this process.

3.7. Involvement of PLD and ERK activity on the LPA-induced cellular proliferation

In order to examine the effect of LPA on the regulation of cell proliferation, we investigated the effect of LPA on [^{3}H]thymidine incorporation in WISH cells. As shown in Fig. 7A, the stimulation of WISH cells by several concentrations of LPA for 24 h induced [^{3}H]thymidine incorporation in a concentration-dependent manner. The stimulation of WISH cells with 10 μM of LPA enhanced this [^{3}H]thymidine incorporation by around two-fold versus the unstimulated control (Fig. 7A). Several studies have reported that PA, a product of phosphatidylcholine hydrolysis by PLD, is involved in the modulation of various cellular functions [21–23]. In this study, we found that LPA stimulates PLD activity in WISH cells (Fig. 3). In order to examine the role of PLD on this LPA-stimulated cell proliferation, we used a well-known PA acceptor, *n*-butanol. LPA stimulated the formation of PBt not of PA in the presence of 0.5% *n*-butanol, as shown in Fig. 3, whereas PA formation during treatment with LPA was not blocked in the presence of 0.5% *t*-butanol. Fig. 7B shows that the LPA-induced [^{3}H]thymidine incorporation in WISH cells was blocked by 85% in the presence of 0.5% *n*-butanol, but not in the presence of 0.5% *t*-butanol. These results suggest that PA production is essential for [^{3}H]thymidine incorporation by LPA in WISH cells. We also examined the effect of MAPKs (ERK and p38 kinase) on LPA-induced cell proliferation in WISH cells. As shown in Fig. 7B, LPA-induced [^{3}H]thymidine incorporation was almost totally inhibited in the presence of PD98059 but not in the presence of SB203580. These results suggest that ERK, not p38 kinase, is essential for LPA-induced [^{3}H]thymidine incorporation by WISH cells.

4. Discussion

Though some reports have shown that LPA is present in the follicular fluid and have suggested that LPA is a biological mediator of normal ovarian physiology, the expressions of LPA receptors and the role of LPA in human amnion-derived cells have not fully investigated. In the present study, we found that human amnion-derived WISH cells express two LPA receptors, LPA_1 and LPA_2, but not

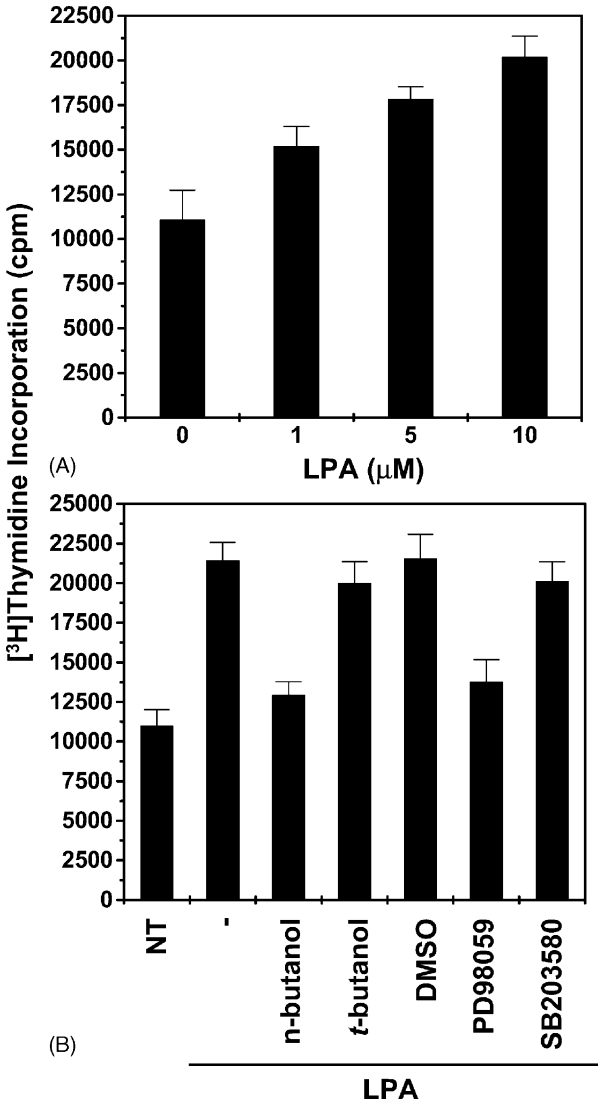

Fig. 7. Effect of LPA on the cellular proliferation of WISH cells. WISH cells (5×10^4) were treated with various concentrations of LPA for 24 h (A), or cells were preincubated with vehicle, 50 μM PD98059 (15 min), 20 μM SB203580 (15 min), 0.5% *n*-butanol (1 min), or 0.5% *t*-butanol (1 min) prior to being treated with 10 μM of LPA for 24 h (B). Cells were harvested and [^{3}H]thymidine incorporation was measured. The results shown are representative of three independent experiments performed in triplicate (A, B).

LPA_3, which indicates that LPA may modulate the cellular activity of WISH cells. To identify the functional role of LPA stimulation in WISH cells, we focused on gene induction and cell proliferation. As shown in Figs. 5 and 7, LPA potently stimulated the induction of two early genes (c-Fos and c-Jun) and cell proliferation. Since amnion cell proliferation is important for the maintenance of pregnancy, these data in WISH cells suggests that LPA plays a crucial role in the maintenance of pregnancy.

Previous reports have demonstrated that LPA binds to several isoforms of cell surface receptors, namely, LPA_1, LPA_2, and LPA_3 [7,8]. When we performed RT-PCR to examine the expression patterns of LPA receptors on WISH cells, we found that WISH cells express LPA_1, LPA_2, but not LPA_3 (Fig. 1). According to previous reports, LPA_1 and LPA_2 interact with G proteins of the $G_{i/o}$, $G_{q/11/14}$, and $G_{12/13}$ families, whereas LPA_3 combines with $G_{i/o}$ and $G_{q/11/14}$ proteins [24–26]. In our study, we investigated the effect of PTX, (which specifically inactivates $G_{i/o}$-mediated signaling pathways) on LPA-induced signaling. When WISH cells were pretreated with 100 ng/ml of PTX for 24 h prior to LPA stimulation, LPA-induced c-Fos and c-Jun expressions were not inhibited (Fig. 6A), and LPA-stimulated ERK activation was not blocked by PTX pretreatment, as shown in Fig. 6B. These results suggest that LPA modulates ERK activation and leads to early gene induction, and that PTX-insensitive G-protein coupled receptors are involved in this process in WISH cells.

To investigate the signal pathway of the early gene induction by LPA in WISH cells, we examined the role of these MAPKs. We observed that LPA stimulated both ERK and p38 kinase activity (Fig. 4A). To determine the role of ERK or p38 kinase on LPA-induced early gene expression, we pretreated WISH cells with two different MAPK-selective inhibitors, i.e., PD98059 and SB203580 (selective ERK and p38 kinase inhibitors, respectively). The preincubation of WISH cells with PD98059 prior to LPA stimulation blocked c-Fos and c-Jun expression by LPA (Fig. 5B); however, SB203580, did not affect LPA-induced early gene expression (Fig. 5B). This indicates that ERK, but not p38 kinase, plays a key role in LPA-induced early gene expression in amnion cells.

Our previous report demonstrated that one of the important lipid mediators, sphingosine-1-phosphate (S1P), is present in human-amniotic fluid [16]. Since S1P potently stimulates cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) synthesis [16], we tested the effects of LPA on COX-2 expression and PGE2 synthesis. However, LPA did not potently stimulate COX-2 expression or PGE2 synthesis (data not shown), suggesting that WISH cell activity can be differentially modulated by S1P or LPA. Previous reports have suggested that LPA may modulate cellular activity by inducing the production or secretion of one or more polypeptide hormones [27–29]. To investigate the role of LPA on the regulation of labor, we examined the effect of LPA on the production and secretion of pro-inflammatory cytokines (IL-1β and TNF-α), which are pivotally required for labor response. The stimulation of WISH cells with LPA for various lengths of time did not affect the induction of IL-1β and TNF-α (data not shown). Taken together with the result that LPA did not significantly stimulate PGE2 synthesis, it seems that LPA may not be crucially involved in the regulation of labor in terms of regulating pro-inflammatory aspect of amnion-derived cells.

In conclusion, the present study indicates that LPA acts on the LPA-specific receptor LPA_1 and LPA_2, which are expressed on human amnion-derived WISH cells. Since this study is the first to report on the expression of LPA receptors on human amnion-derived WISH cells, further

studies on the pathophysiological and physiological roles of LPA on the maintenance of labor response are required.

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