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Lysophosphatidic acid stimulates cell proliferation in rat chondrocytes

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Abstract

Rat primary chondrocytes express the lysophosphatidic acid (LPA) receptor, LPA_1, LPA_3, but not LPA_2. When chondrocytes were stimulated with LPA, phospholipase C-mediated cytosolic calcium increase was dramatically induced. LPA also stimulated two kinds of mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK) and p38 kinase in chondrocytes. In terms of the LPA-mediated functional modulation of chondrocytes, LPA stimulated cellular proliferation. We examined the signaling pathways involved in LPA-mediated cellular proliferation. LPA-induced chondrocyte proliferation was almost completely blocked by 2′-amino-3′-methoxyflavone (PD98059) but not by 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), suggesting that ERK activity is essentially required for the process. Pertussis toxin almost completely inhibited the LPA-induced cellular proliferation and ERK activation, indicating the role of $G_{i/o}$ protein(s) in the processes. This study demonstrates the physiological role of LPA on the modulation of rat primary chondrocyte proliferation, and the crucial role played by ERK in the process.

Keywords: Lysophosphatidic acid; Chondrocyte; Proliferation; Pertussis toxin-sensitive G-protein; Extracellular signal-regulated kinase

1. Introduction

Chondrocytes play a key role in the regulation of osteoarthritis [1]. Chondrocytes synthesize cartilage-specific extracellular matrix, such as type II collagen, which is necessary to maintain matrix integrity [2]. In osteoarthritis, this matrix synthesis and degradation homeostasis is damaged, resulting in structural and biochemical changes in chondrocytes and cartilage [1,2]. Since chondrocytes are the only cells resident in mature cartilage, regulations of chondrocyte cell death and proliferation are important for the prevention or treatment of osteoarthritis [3,4].

Abbreviations: BAPTA/AM, 1,2-bis (aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester; $[Ca^{2+}]_i$, intracellular calcium concentration; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated protein kinase; fura-2/AM, fura-2 pentaacetoxymethylester; GPCR, G-protein coupled receptor; IL-1β, interleukin-1β; LPA, lysophosphatidic acid; LY294002, 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; pERK, phospho-ERK; PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C; pp38, phospho-p38; PTX, pertussis toxin; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; $sPLA_2$, secretory phospholipase A2; TCA, trichloroacetic acid; TNF-α, tumor necrosis factor-α; U-73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione

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Several kinds of proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been reported to regulate chondrocyte apoptosis [5,6]. These proinflammatory cytokines increase the number of apoptotic cells by production of nitric oxide via inducible nitric oxide synthase [6]. Moreover, cyclooxygenase-2, which is induced by extracellular signals, plays a key role in the modulation of chondrocyte apoptosis [7]. Several cytokines, such as IL-1β and TNF-α, have been previously shown to induce chondrocyte apoptosis via cyclooxygenase-2 expression [7,8]. Although some cytokines have been reported to regulate chondrocyte apoptosis or proliferation [9–11], further factors involved in chondrocyte activity should be considered.

Lysophosphatidic acid (LPA), an important lipid mediator, has been reported to modulate various (patho)physiological activities in several cell types [12,13]. LPA induces cellular proliferation in primary ovarian carcinoma cells, prostate cancer cell lines, amniotic cells, and so on [14–16]. LPA also regulates the tissue remodeling processes occurring during growth, differentiation, and the regression of the corpus luteum [17]. Previous reports have demonstrated that LPA acts on plasma membrane-bound cell surface receptors, a family of G-protein coupled receptor (GPCR), such as LPA_1, LPA_2, and LPA_3 [18,19]. Although many previous reports have demonstrated the pivotal role of LPA in the modulation of several biological responses, the expression of LPA receptors in chondrocytes and the role of LPA in these cells have not been previously investigated. Many reports have demonstrated the involvement of lipid factors in cellular responses. In particular, it is reasonable to assume that lipid ligands might be involved in the regulation of chondrocyte activity in combination with several kinds of protein factor.

In this study, we investigated whether LPA receptors are expressed on rat primary chondrocytes and LPA modulate cellular activity of the cells. We also investigated the signaling pathways involved in the LPA-mediated chondrocyte activation.

2. Materials and methods

2.1. Reagents

LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate, sodium salt) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). The reverse transcription-polymerase chain reaction kit was purchased from Invitrogen Corporation (Carlsbad, CA), and fetal bovine serum from Hyclone (Logan, UT). Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ) and phospho-ERK1/2, phospho-p38 kinase, and ERK2 antibodies from New England Biolabs (Beverly, MA). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexyl]-1*H*-pyrrole-2,5-dione (U-73122), 1,2-bis (aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester (BAPTA/AM), 2′-amino-3′-methoxyflavone (PD98059), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) 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

Rat articular chondrocytes were released from cartilage slices of 6-week-old Sprague–Dawley rats by enzymatic digestion as described previously [20]. Briefly, cartilage slices were aseptically dissected and then dissociated enzymatically for 6 h in 0.2% collagenase type II (125 U/mg solid; Sigma) in phosphate-buffered saline. Individual cells were then obtained by collecting the supernatant after brief centrifugation. Isolated rat primary chondrocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. The cells were subcultured twice weekly by trypsinization and seeded in either 12-well (2×10^5 cells/well) or 6-well plates (5×10^5 cells/well). This study was approved by local institutional review boards at Dong-A University Hospital.

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 [21]. 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 pentaa-

cetoxymethylester (fura-2/AM) [22]. 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 nm and 380 nm, and the calculated fluorescence ratio was translated into $[Ca^{2+}]_i$.

2.5. Western blot analysis

Rat primary chondrocytes 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 × *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 ECL system.

2.6. Cellular proliferation assay

Rat primary chondrocytes were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/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/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.

2.7. Statistics

The results are expressed as means ± S.E. of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $p < 0.05$.

3. Results

3.1. Expression of LPA receptors in rat primary chondrocytes

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

3.2. LPA stimulates $[Ca^{2+}]_i$ increase in rat primary chondrocytes

Previously, it has been demonstrated that the activation of LPA-specific receptors by LPA binding causes diverse intracellular signals, including $[Ca^{2+}]_i$ increase [23]. To confirm that LPA receptors on rat primary chondrocytes are functional, we examined the effect of LPA upon $[Ca^{2+}]_i$ in rat primary chondrocytes. As shown in Fig. 2A, the stimulation of rat primary chondrocytes with 5 μM of LPA caused a $[Ca^{2+}]_i$ increase in the presence or in the absence of extracellular calcium. Concentration-dependency of LPA-induced $[Ca^{2+}]_i$ increase was also investigated in rat primary chondrocytes. Increase of $[Ca^{2+}]_i$ was apparent at 100 nM of LPA and maximal activity was observed at 5–10 μM concentration (Fig. 2B).

3.3. LPA-induced $[Ca^{2+}]_i$ increase is mediated by pertussis toxin-sensitive G-proteins and phospholipase C activation

One of the well-known mechanism of $[Ca^{2+}]_i$ increase in the absence of extracellular calcium is the phospholipase C

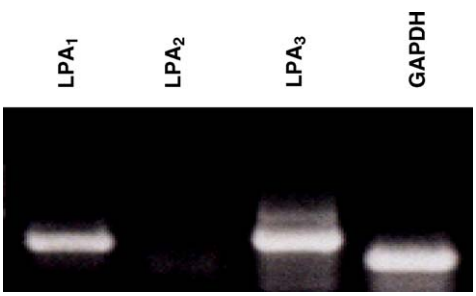

Fig. 1. Expression of LPA receptors in rat primary chondrocytes. RT-PCR analysis was performed on mRNA isolated from cultured rat primary chondrocytes. The data presented are representative of three independent experiments.

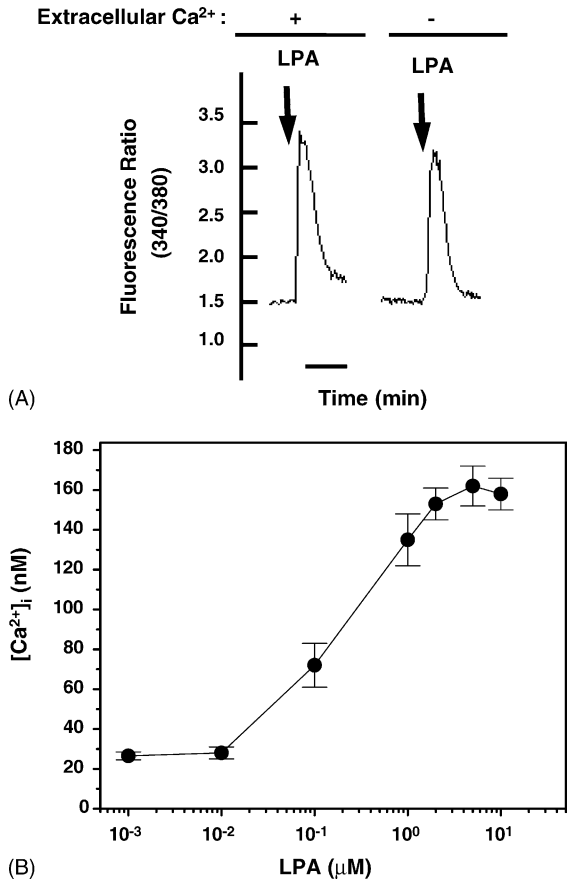

Fig. 2. The effect of LPA on intracellular calcium increase in rat primary chondrocytes. Rat primary chondrocytes were stimulated with 5 μM of LPA, and $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM. Peak $[Ca^{2+}]_i$ levels were recorded (A). Rat primary chondrocytes were stimulated by various concentrations of LPA. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as means ± S.E. of three independent experiments, which performed in duplicate (B).

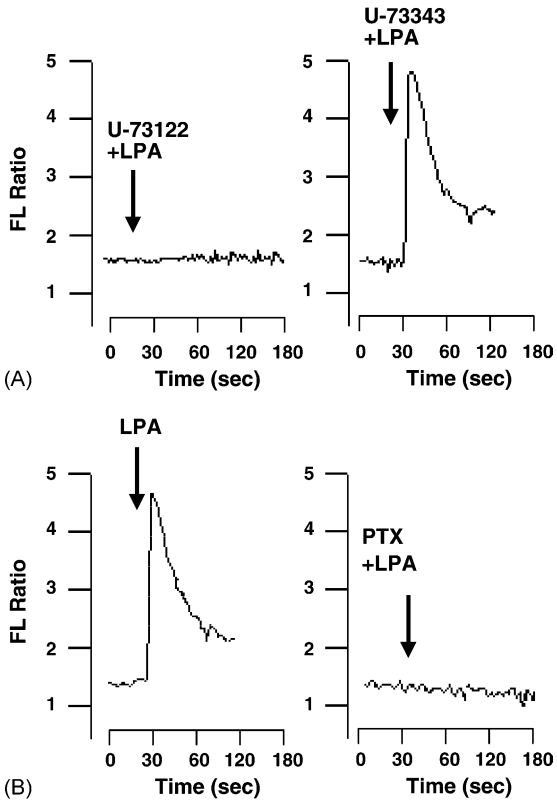

Fig. 3. LPA-induced Ca^{2+} signaling is PLC- and PTX-sensitive G-proteins mediated in rat primary chondrocytes. Rat primary chondrocytes were pretreated with 5 μM of U-73122 or 5 μM of U-73343 prior to 5 μM of LPA, and $[Ca^{2+}]_i$ was determined (A). Rat primary chondrocytes were preincubated in the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

(PLC)-dependent inositol-1,4,5-trisphosphate-mediated response [24]. To determine the role of 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. 3A 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 rat primary chondrocytes. We also examined the effect of pertussis toxin (PTX), a specific inhibitor of $G_{i/o}$ type G-proteins, on LPA-induced $[Ca^{2+}]_i$ increase. When rat primary chondrocytes were preincubated with 100 ng/ml of PTX prior to being stimulated with 5 μM LPA, LPA-induced $[Ca^{2+}]_i$ increase was completely inhibited (Fig. 3B). These results indicate that LPA stimulates $[Ca^{2+}]_i$ increase via PTX-sensitive pathway. As a negative control, we found that ATP also stimulated $[Ca^{2+}]_i$ increase in rat primary chondrocytes (data not shown), and ATP-stimulated $[Ca^{2+}]_i$ increase was not affected by PTX, indicating that ATP stimulates $[Ca^{2+}]_i$ increase in a PTX-insensitive manner in the cells.

3.4. LPA stimulates mitogen-activated protein kinases in rat primary chondrocytes

Mitogen-activated protein kinase (MAPK) has been reported to mediate extracellular signals to the nucleus in various cell types [25]. In this study, we examined whether LPA stimulates MAPKs by Western blotting with anti-phospho-specific antibodies to each enzyme. When rat primary chondrocytes 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 5–10 min of stimulation (Fig. 4A). Another important MAPK, p38 kinase, was also transiently phosphorylated by LPA stimulation with kinetics that resembled those of ERK phosphorylation (Fig. 4A). We also found that stimulation of rat primary chondrocytes with various concentrations of LPA-induced MAPK phosphorylation. LPA-induced ERK phosphorylation in a concentration-dependent manner, showing maximal activity at 100 nM (Fig. 4B). p38 kinase phosphorylation was also induced with a similar concentration

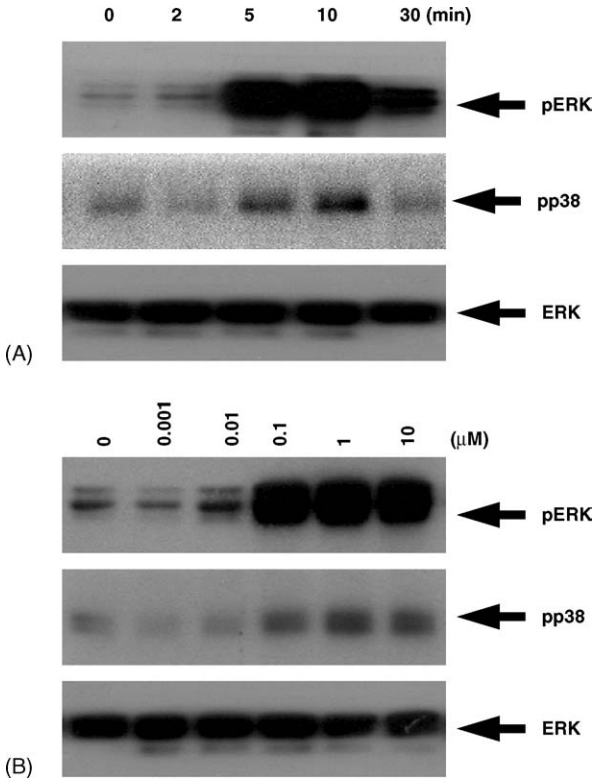

Fig. 4. Activation of MAPKs by LPA in rat primary chondrocytes. Rat primary chondrocytes were stimulated with 5 μM of LPA for various times (A). The cells were stimulated with various concentrations of LPA for 10 min (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phospho-ERK (pERK) or phospho-p38 (pp38) 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 (A and B).

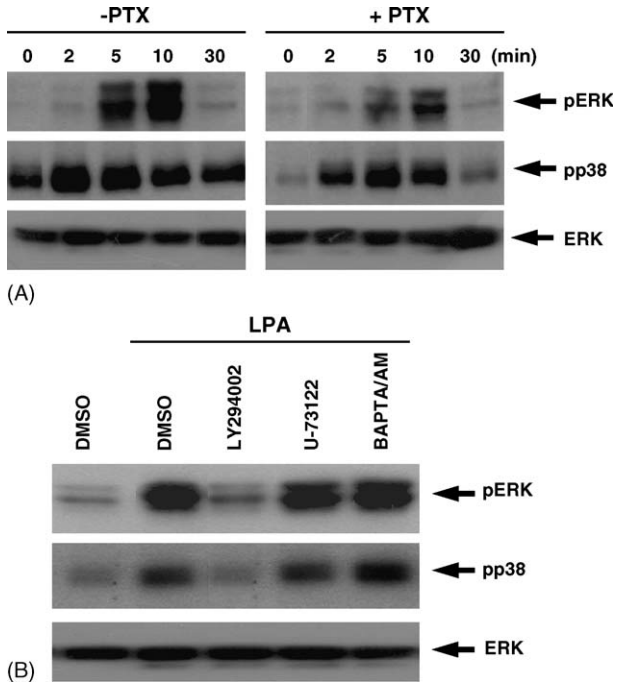

Fig. 5. Regulation of LPA-induced MAPK phosphorylation in rat primary chondrocytes. Rat primary chondrocytes were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were stimulated with 5 μM of LPA for several times (A). Rat primary chondrocytes were preincubated with LY294002 (50 μM), U-73122 (5 μM), or BAPTA/AM (10 μM) for 15 min prior to adding 5 μM LPA for 10 min (B). Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and the phospho-ERK (pERK) or phospho-p38 (pp38) kinase were determined by immunoblotting using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody (B). The results shown are representative of at least three independent experiments (A and B).

dependency that resembled those of p38 kinase phosphorylation (Fig. 4B).

3.5. Regulation of LPA-induced MAPK activation

We investigated the role of PTX-sensitive G-protein on LPA-induced MAPK activation. Cultured rat primary chondrocytes were preincubated with 100 ng/ml of PTX prior to being stimulated with 5 μM LPA. We found that pretreatment with PTX dramatically blocked ERK phosphorylation by LPA (Fig. 5A), showing that LPA induces ERK activation in a PTX-sensitive manner. Preincubation of rat primary chondrocytes with 100 ng/ml of PTX slightly inhibited p38 kinase phosphorylation (Fig. 5A). The result suggests that LPA stimulates p38 kinase activity in both of PTX-sensitive and PTX-insensitive pathways. We also examined the signaling pathway of LPA-induced MAPKs phosphorylation by stimulating rat primary chondrocytes with LPA in the presence of several inhibitors of some signaling molecules for 15 min. LY294002 and U-73122 is a selective inhibitor for phosphatidylinositol-3-kinase (PI3K) and PLC, respectively [26,27]. BAPTA/AM is a Ca^{2+} chelator [28]. As shown in Fig. 5B, LY294002 inhibited LPA-induced ERK phosphorylation, but U-73122 and BAPTA/AM did not (Fig. 5B). These results indicate that LPA stimulates ERK phosphorylation via a PI3K-mediated pathway. LPA-induced p38 kinase phosphorylation was also inhibited by LY294002 but not by U-73122 and BAPTA/AM (Fig. 5B), suggesting PI3K-dependency.

3.6. LPA induces chondrocyte proliferation via ERK pathway

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 rat primary chondrocytes. As shown in Fig. 6A, the stimulation of rat primary chondrocytes by several concentrations of LPA for 24 h induced [^{3}H]thymidine incorporation in a concentration-dependent manner. The stimulation of rat primary chondrocytes with 10 μM of LPA enhanced this [^{3}H]thymidine incorporation by around five-fold versus the unstimulated control (Fig. 6A). We also examined the effect of PTX on LPA-induced chondrocyte proliferation. When rat primary chondrocytes were preincubated with 100 ng/ml of PTX

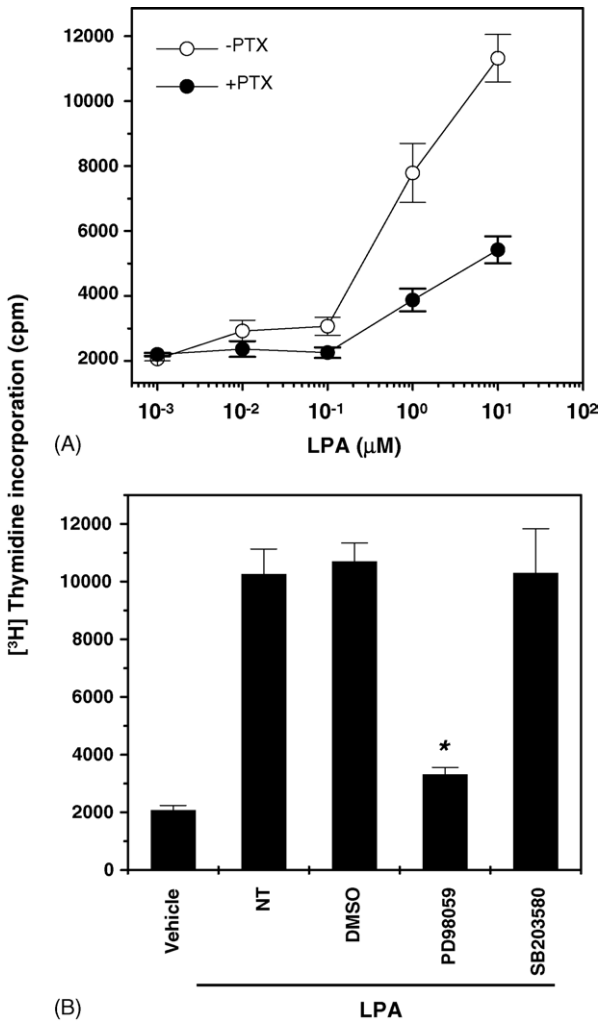

Fig. 6. Effect of LPA on chondrocyte proliferation. Rat primary chondrocytes (5×10^4) were treated with various concentrations of LPA for 24 h in the absence or presence of 100 ng/ml of PTX (A). The cells were pre-incubated with vehicle, 50 μM PD98059 (15 min), or 20 μM SB203580 (15 min) prior to being treated with 10 μM of LPA for 24 h (B). Cells were harvested and [^{3}H]thymidine incorporation was measured. Vehicle is distilled water and NT indicates not treated. Results are presented as means ± S.E. of three independent experiments, which performed in duplicate (A and B). Asterisk (*) indicates results significantly different at the $p < 0.05$ probability levels as compared to the values obtained from the control (DMSO treated).

prior to stimulation with LPA, LPA-induced [^{3}H]thymidine incorporation was dramatically inhibited (Fig. 6A), strongly suggesting the involvement of PTX-sensitive G-proteins.

Several studies have reported that ERK activity is involved in the modulation of various cellular functions, such as cell proliferation [29,30]. In this study, we found that LPA stimulates ERK activity in rat primary chondrocytes (Fig. 4). In order to examine the role of ERK on this LPA-stimulated cell proliferation, we used a well-known ERK pathway inhibitor, PD98059. The ERK pathway inhibitor PD98059 [31] prevents activation of mitogen-activated protein kinase (MEK)1/2 [32], upstream activators of ERK 1/2, whereas the pyridinyl imidazole SB203580 inhibits p38 kinase activity [32,33]. Fig. 6B shows that the LPA-induced [^{3}H]thymidine incorporation in rat primary chondrocytes was blocked by 85% in the presence of 50 μM of PD98059, but not in the presence of 20 μM of SB203580. These results suggest that ERK activity is essential for [^{3}H]thymidine incorporation by LPA in rat primary chondrocytes.

4. Discussion

In arthritis, chondrocyte death and apoptosis have been regarded as an important cause of arthritis development [1–4]. Various kinds of mediators have been reported to regulate chondrocyte apoptosis. They include Fas and Fas ligand, nitric oxide, and inflammatory cytokines, such as TNF-α and IL-1β [5–8]. Many groups have reported that several kinds of inflammatory cytokines are involved in the regulation of chondrocyte death or proliferation [5–8]. However, the role of GPCR ligands on the modulation of chondrocyte proliferation has not been studied. In this study, we demonstrated for the first time that LPA stimulates cell proliferation in rat primary chondrocytes (Fig. 6). The role of LPA on the modulation of cell proliferation has already been reported in other cell types [34–36]. It has been demonstrated that LPA stimulates and leads to proliferative activity in fibroblasts [36]. Our study indicates that LPA also regulates cell proliferation in rat primary chondrocytes, suggesting that it has a role in the modulation of arthritis. Moreover, this finding strongly suggests not only proinflammatory cytokines, such as TNF-α and IL-1β but also GPCR ligands like LPA might be involved in the regulation of chondrocyte functioning. Since rat primary chondrocytes proliferation is important for the therapy of arthritis, these data in rat primary chondrocytes suggest that LPA can be regarded as a crucial target for the therapy of arthritis.

LPA has been reported to bind to several isoforms of cell surface receptors, namely LPA_1, LPA_2, and LPA_3 [18,19]. When we performed RT-PCR to examine the expression patterns of LPA receptors on rat primary chondrocytes, we found that rat primary chondrocytes express LPA_1, LPA_3, but not LPA_2 (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 [37–39]. In our study, we investigated the effect of PTX (which specifically inactivates $G_{i/o}$-mediated signaling pathways) on LPA-induced signaling. When rat primary chondrocytes were pretreated with 100 ng/ml of PTX for 24 h prior to LPA stimulation, LPA-induced cell proliferation was dramatically inhibited (Fig. 6A), and LPA-stimulated ERK activation was also blocked by PTX pretreatment, as shown in Fig. 5A. These results suggest that LPA modulates ERK activation and leads to cell proliferation, and that PTX-sensitive GPCRs

are involved in these processes in rat primary chondrocytes.

On the role of two MAPKs on the cell proliferation by LPA in rat primary chondrocytes, we observed that ERK, but not p38 kinase, plays a key role. On the regulation of LPA-induced MAPKs activation, we found that PI3K but not PLC-mediated Ca^{2+} plays a role in rat primary chondrocytes (Fig. 5B). Since LPA stimulated PI3K-mediated Akt phosphorylation in rat primary chondrocytes and this LPA-induced Akt phosphorylation was completely blocked by PTX (data not shown), it will be reasonable to assume that LPA stimulates its specific receptor(s) resulting in PTX-sensitive G-protein(s)/PI3K/ERK pathway which is essential for the proliferative activity in the cells. In Fig. 3, we demonstrated that LPA stimulates $[Ca^{2+}]_i$ increase in a PTX-sensitive manner. Taken together the signaling pathways of LPA, it is appears that LPA stimulates at least two different G-protein coupled signalings; i.e., PLC-mediated $[Ca^{2+}]_i$ increase and PI3K-mediated MAPKs activation via PTX-sensitive G-protein-mediated pathways in rat primary chondrocytes. In view of the fact that calcium signaling regulates various kinds of cellular physiologies and that LPA dramatically stimulates PLC-mediated $[Ca^{2+}]_i$ increase, it would be interesting to know the other functional roles of LPA in rat primary chondrocytes related to calcium signaling-dependent processes.

Previously it has been reported that secretory phospholipase A_2 ($sPLA_2$), which hydrolyzes the sn-2 position of glycerophospholipids and liberates free fatty acids (mainly arachidonic acid) and lysophospholipids such as LPA, was markedly elevated in 25% of 51 patients with arthritis and that synovial fluid contained high levels of $sPLA_2$ activity [40]. Furthermore, several cell types including chondrocytes found to produce and secrete $sPLA_2$, and synovial fluid contains good substrate for the hydrolysis of $sPLA_2$ [41,42]. These results suggest the role of the enzymatic activity of $sPLA_2$ and the product (LPA) might be associated with arthritis pathogenesis. However, the role of LPA on the functional regulation of chondrocyte activity was not investigated yet. The present study indicates that LPA stimulates rat primary chondrocytes, resulting in cell proliferation via the LPA-specific receptors. Very recently, Flamigni and coworkers reported that LPA exert anti-proliferative effect in immortalized human juvenile costal chondrocytes, T/C-28a2 [43]. At this point, it is unclear what caused this differential effect on the cell proliferative activity by LPA in chondrocytes. It should be examined whether LPA induce differential signaling and functional activity in rat primary chondrocytes or in immortalized human chondrocytes. Intracellular detail signaling pathways involved in anti-proliferative or proliferative effects of LPA also should be compared. Since this study is the first to report on the expression of LPA receptors on rat primary chondrocytes, further studies on the (patho)physiological roles of LPA on the cause of arthritis and therapeutic target for arthritis are required.

Acknowledgements

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