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Sphingosine-1-phosphate stimulates rat primary chondrocyte proliferation

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Abstract

Rat primary chondrocytes express the sphingosine-1-phosphate (S1P) receptor, $S1P_2$, $S1P_3$, $S1P_4$, but not $S1P_1$. When chondrocytes were stimulated with S1P or phytosphingosine-1-phosphate (PhS1P, an $S1P_1$- and $S1P_4$-selective agonist), phospholipase C-mediated cytosolic calcium increase was dramatically induced. S1P and PhS1P also stimulated two kinds of mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK) and p38 kinase in chondrocytes. In terms of the two phospholipids-mediated functional modulation of chondrocytes, S1P and PhS1P stimulated cellular proliferation. The two phospholipids-induced chondrocyte proliferations were almost completely blocked by PD98059 but not by SB203580, suggesting that ERK but not p38 kinase is essentially required for the proliferation. Pertussis toxin almost completely inhibited the two phospholipids-induced cellular proliferation and ERK activation, indicating the crucial role of G_i protein. This study demonstrates the physiological role of two important phospholipids (S1P and PhS1P) on the modulation of rat primary chondrocyte proliferation, and the crucial role played by ERK in the process.

Keywords: Sphingosine-1-phosphate; Phytosphingosine-1-phosphate; Chondrocyte; Proliferation; Pertussis toxin-sensitive G-protein; Extracellular signal-regulated kinase

Chondrocytes which synthesize cartilage-specific extracellular matrix, such as type II collagen, maintain matrix integrity [1]. Chondrocytes are also key regulators in osteoarthritis [1]. In osteoarthritis, this matrix synthesis and degradation homeostasis is damaged, resulting in structural and biochemical changes in chondrocytes and cartilage [1,2]. It is regarded that chondrocyte cell death and proliferation are important for the prevention or treatment of osteoarthritis [3,4]. Previous reports demonstrated that many proinflammatory cytokines, including interleukin-1β and tumor necrosis factor-α, regulate chondrocyte apoptosis [5,6]. Although some cytokines have been reported to regulate chondrocyte apoptosis or proliferation [5,6], further factors involved in chondrocyte activity should be considered.

Sphingosine-1-phosphate (S1P), an important lipid mediator, has been reported to modulate various (patho)physiological activities in several cell types [7,8]. S1P induces chemotactic migration and angiogenesis in human umbilical vein endothelial cells [9]. S1P induces cellular chemotaxis and modulates cytokine release in mature human dendritic cells emerging of Th2 immune responses [10] and in human bronchial epithelial cells. On the cell surface receptors for S1P, a family of G-protein coupled receptors ($S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$) has been reported [8,11–13]. Although many previous reports have demonstrated the pivotal role of S1P in the modulation of several biological responses via its specific receptor, the role of S1P in chondrocytes has not been previously studied.

Phytosphingosine-1-phosphate (PhS1P) can be generated by phosphorylation of phytosphingosine which is one of the most widely distributed natural sphingoid bases abundantly in fungi and plants, and also found in animals including human [14,15]. PhS1P is structurally similar to

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S1P; PhS1P has a hydroxyl group at C-4 of the sphingoid long-chain base, where S1P has a *trans*-double bond between C-4 and C-5. Although PhS1P is found in animals as a minor component of cell membrane, the physiological role of PhS1P in the modulation of cellular activities has not been previously studied.

In this study, we investigated whether S1P receptors are expressed on rat primary chondrocytes, and S1P and PhS1P modulate cellular activity of the cells. We also investigated the signaling pathways involved in the S1P- and PhS1P-mediated chondrocyte activation.

Materials and methods

Materials. S1P and PhS1P were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). The reverse transcription-polymerase chain reaction kit and fetal bovine serum were purchased from Invitrogen Corporation (Carlsbad, CA). 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.

Cell culture. Rat articular chondrocytes were released from cartilage slices of 6-week-old Sprague–Dawley rats by enzymatic digestion as described previously [16]. 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- (2×10^5 cells/well) or 6-well plates (5×10^5 cells/well).

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 sequences of the primer used were as follows; $S1P_1$ (217 bp product): forward, 5′-ACCCTAGCATTTCAAAAACA-3′; reverse, 5′-AAACGACTGAAGGAAGTGAA-3′. $S1P_2$ (291 bp product): forward, 5′-AGTGACAAAAGCTGTCGAAT-3′; reverse, 5′-TACGA TGGTGACTGTCTTGA-3′. $S1P_3$ (258 bp product): forward, 5′-CCTTT CTCAGCATCTTC-3′; reverse, 5′-TGATGAACCACTGACTCTTG-3′. $S1P_4$ (265 bp product): forward, 5′-AACATCAGTACCTGGTCCAC-3′; reverse, 5′-TCAGGAGGCAGTACACC-3′. GAPDH (246 bp product): forward, 5′-GATGACATCAAGAAGGTGGTGAA-3′, reverse, 5′-GTC TTACTCCTTGGAGGCCATGT-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.

$[Ca^{2+}]_i$ measurements. Intracellular calcium concentration $[Ca^{2+}]_i$ was determined using Grynkiewicz's method using fura-2/AM [17,18]. 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 and at excitation wavelengths of 340 and 380 nm, and the calculated fluorescence ratio was translated into $[Ca^{2+}]_i$.

Western blot analysis. Rat primary chondrocytes were plated in a 6-well plate and treated with S1P or PhS1P 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 dried 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 ECLsystem.

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 S1P or PhS1P treatment, the cells were seeded in 24-well plates, in triplicate, at 5×10^4 cells/well. Complete medium was replaced with serum-free RPMI 1640 medium to starve the cells for 24 h. S1P or PhS1P 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.

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

Results

Expression of S1P receptors in rat primary chondrocytes

Previously we reported that rat primary chondrocytes express LPA receptors [19], and in this study, we investigated whether cell surface receptors for S1P are expressed on rat primary chondrocytes. To determine which S1P receptor isoforms are expressed on rat primary chondrocytes, we analyzed the mRNA expressions of different S1P receptors by semi-quantitative RT-PCR. As shown in Fig. 1, rat primary chondrocytes expressed three isoforms of S1P receptor, namely, $S1P_2$, $S1P_3$, and $S1P_4$ (Fig. 1); we were unable to detect $S1P_1$ expression (Fig. 1). We confirmed

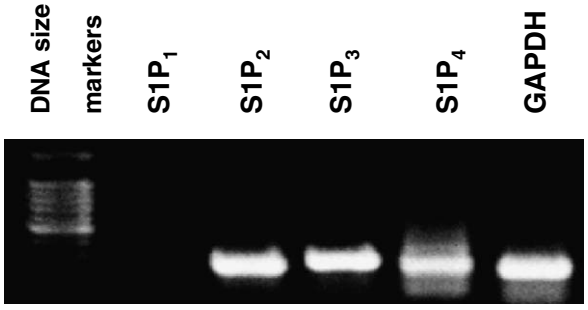

Fig. 1. Expression of S1P receptors in rat primary chondrocytes. RT-PCR analysis was performed on mRNA isolated from cultured rat primary chondrocytes. The data presented are representative of three independent experiments.

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 $S1P_2$, $S1P_3$, and $S1P_4$.

Stimulation of S1P receptors induces $[Ca^{2+}]_i$ increase in rat primary chondrocytes

Previously it has been demonstrated that the activation of S1P-specific receptors by S1P binding causes diverse intracellular signals, including $[Ca^{2+}]_i$ increase [20]. To confirm that S1P receptors on rat primary chondrocytes are functional, we examined the effect of S1P upon $[Ca^{2+}]_i$ in rat primary chondrocytes. As shown in Fig. 2A, the stimulation of rat primary chondrocytes with 2 μM S1P caused a $[Ca^{2+}]_i$ increase in the presence or in the absence of extracellular calcium. According to previous report PhS1P has been reported as a high affinity ligand for $S1P_4$ [21]. Since rat primary chondrocytes express $S1P_4$, we tested the effect of PhS1P on $[Ca^{2+}]_i$ increase in the cells. Stimulation of rat primary chondrocytes with 2 μM PhS1P elicited a dramatic $[Ca^{2+}]_i$ increase (Fig. 2A). Concentration-dependency of S1P- or PhS1P-induced $[Ca^{2+}]_i$ increase was also investigated in rat primary chondrocytes. Increase of $[Ca^{2+}]_i$ was apparent at 1 nM S1P and maximal activity was observed at 100 nM concentration (Fig. 2B). PhS1P also stimulated $[Ca^{2+}]_i$ increase showing a concentration-dependency (Fig. 2B).

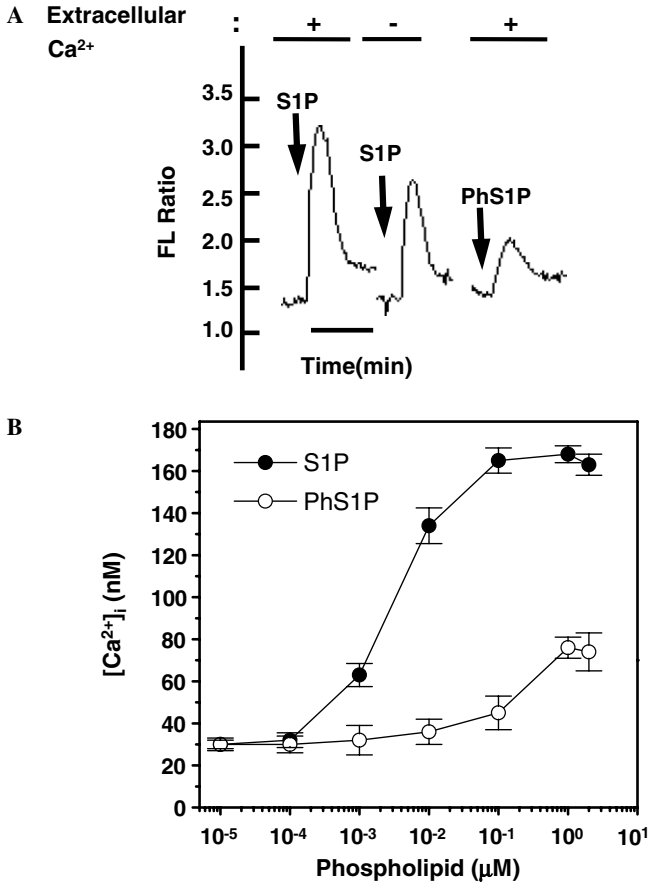

Fig. 2. The effect of S1P and PhS1P on intracellular calcium increase in rat primary chondrocytes. Rat primary chondrocytes were stimulated with 2 μM S1P or 2 μM PhS1P, and $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM. Peak $[Ca^{2+}]_i$ levels were recorded (A). Rat primary chondrocytes were stimulated by various concentrations of S1P or PhS1P. The peak level of $[Ca^{2+}]_i$ was recorded. Results are presented as means ± SE of three independent experiments, which were performed in duplicate (B).

PhS1P- but not S1P-induced $[Ca^{2+}]_i$ increase is mediated by pertussis toxin-sensitive G-proteins

To determine the role of phospholipase C (PLC) on S1P-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 S1P-induced $[Ca^{2+}]_i$ increase. This result indicates that S1P 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 S1P-induced $[Ca^{2+}]_i$ increase. When rat primary chondrocytes were preincubated with 100 ng/ml PTX prior to being stimulated with 2 μM S1P, S1P-induced $[Ca^{2+}]_i$ increase was not affected (Fig. 3B). These results indicate that S1P stimulates $[Ca^{2+}]_i$ increase via PTX-insensitive pathway. We also examined the signaling for the PhS1P-induced $[Ca^{2+}]_i$ increase. PhS1P-induced $[Ca^{2+}]_i$ increase was also completely inhibited by U-73122 (Fig. 3C). In contrast to S1P-induced calcium release, PhS1P-stimulated $[Ca^{2+}]_i$ increase was completely inhibited by PTX, indicating that PhS1P stimulates $[Ca^{2+}]_i$ increase in a PTX-sensitive manner in the cells (Fig. 3D).

S1P and PhS1P stimulate mitogen-activated protein kinases in rat primary chondrocytes

We examined whether S1P stimulates mitogen-activated protein kinases (MAPKs) by Western blotting with anti-phospho-specific antibodies to each enzyme. When rat primary chondrocytes were stimulated with 2 μM S1P 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 S1P stimulation with kinetics that resembled those of ERK phosphorylation (Fig. 4A). We also found that stimulation of rat primary chondrocytes with various concentrations of S1P-induced MAPK phosphorylation. S1P-induced ERK phosphorylation in a concentration-dependent manner, showing maximal activity at 100 nM (Fig. 4B). P38 kinase phosphorylation was also induced in a concentration-dependent manner, showing maximal activity at 2 μM (Fig. 4B). We also examined the effect of PhS1P on the two MAPKs activation. PhS1P stimulated not only ERK

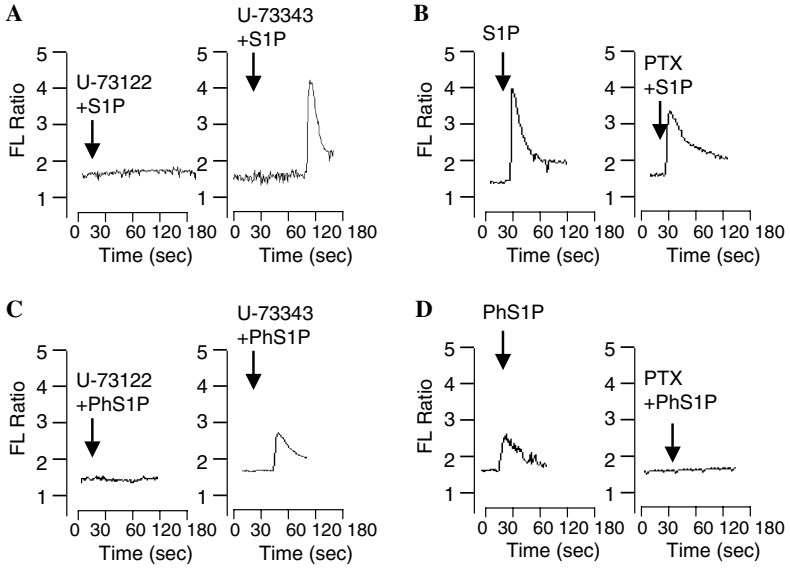

Fig. 3. Effect of PLC inhibitor and PTX on S1P- or PhS1P-induced Ca^{2+} signaling in rat primary chondrocytes. Rat primary chondrocytes were pretreated with 5 μM U-73122 or 5 μM U-73343 prior to 2 μM S1P (A) or 2 μM PhS1P (C). Rat primary chondrocytes were preincubated in the absence or presence of 100 ng/ml PTX for 24 h. Rat primary chondrocytes were then loaded with fura-2/AM and $[Ca^{2+}]_i$ was determined fluorometrically after stimulation with 2 μM S1P (B) or 2 μM PhS1P (D). Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of four independent experiments (A–D).

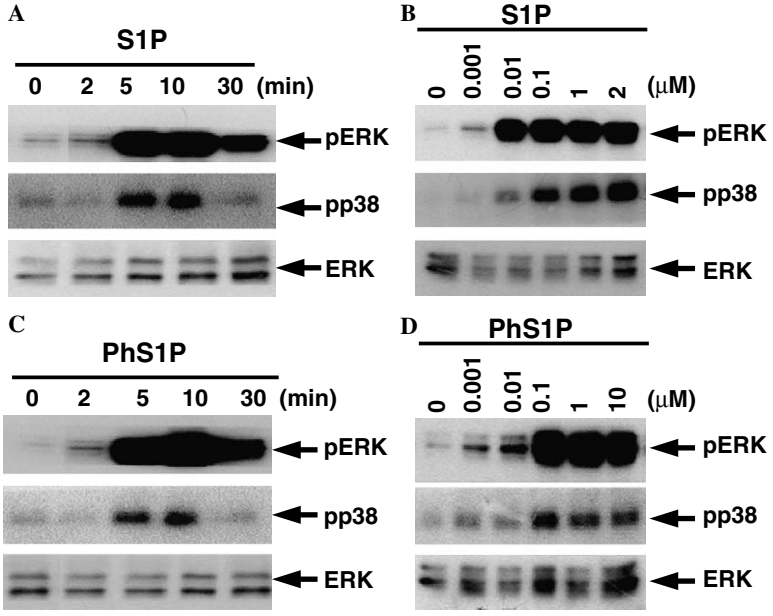

Fig. 4. Activation of MAPKs by S1P or PhS1P in rat primary chondrocytes. Rat primary chondrocytes were stimulated with 2 μM S1P (A) or 2 μM PhS1P (C) for various times. The cells were stimulated with various concentrations of S1P (B) or PhS1P (D) for 10 min. Each sample (30 μg of protein) was subjected 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–D).

but also p38 kinase in a similar time- and concentration-dependent manner (Figs. 4C and D).

Regulation of S1P- or PhS1P-induced MAPK activation

The role of PTX-sensitive G-protein on S1P-induced MAPK activation was investigated. Cultured rat primary chondrocytes were preincubated with 100 ng/ml PTX prior to being stimulated with 2 μM S1P. We found that pretreatment with PTX dramatically blocked ERK phosphorylation by S1P (Fig. 5A), showing that S1P induces ERK activation in a PTX-sensitive manner. Preincubation of rat primary chondrocytes with 100 ng/ml PTX slightly inhibited p38 kinase phosphorylation (Fig. 5A). The result

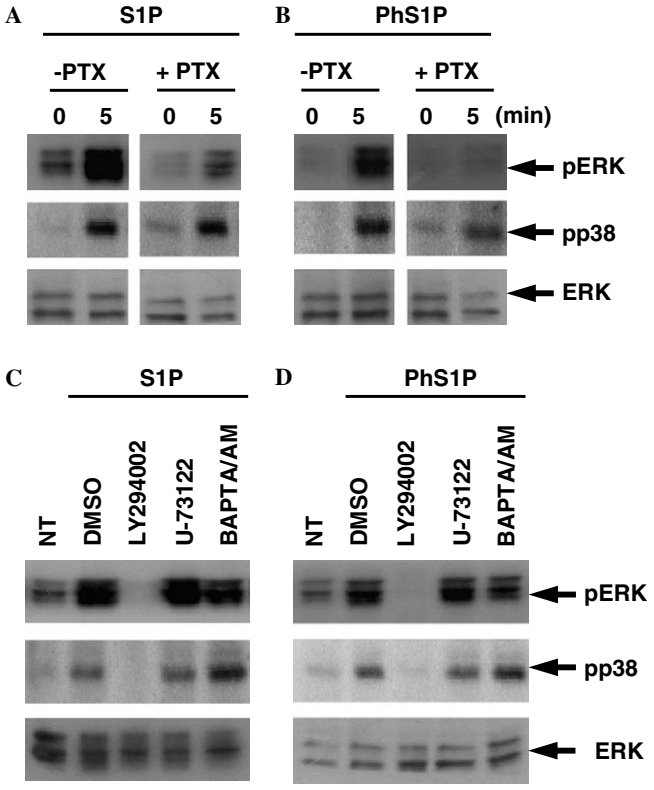

Fig. 5. Regulation of S1P- or PhS1P-induced MAPK phosphorylation in rat primary chondrocytes. Rat primary chondrocytes were preincubated in the absence or presence of 100 ng/ml PTX for 24 h. Cells were stimulated with 2 μM S1P (A) or 2 μM PhS1P (B) for 5 min (A). Rat primary chondrocytes were preincubated with LY294002 (50 μM), U-73122 (5 μM), or BAPTA/AM (10 μM) for 15 min (60 min for BAPTA/AM) prior to adding 2 μM S1P (C) or 2 μM of PhS1P (D) 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 was determined by immunoblotting using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody (A–D). The results shown are representative of at least three independent experiments (A–D).

suggests that S1P stimulates p38 kinase activity in both of PTX-sensitive and PTX-insensitive pathways. PhS1P-induced ERK and p38 kinase phosphorylations were almost completely inhibited by PTX (Fig. 5B). It indicates that PhS1P stimulates ERK and p38 kinase in a PTX-sensitive manner. We also examined the signaling pathway of S1P-induced MAPKs phosphorylation by stimulating rat primary chondrocytes with S1P in the presence of several inhibitors of some signaling molecules. As shown in Fig. 5B, LY294002 (a selective inhibitor for phosphatidylinositol-3-kinase (PI3K)) inhibited S1P-induced ERK phosphorylation, but U-73122 (a PLC inhibitor) and BAPTA/AM (a calcium chelator) did not (Fig. 5C). These results indicate that S1P stimulates ERK phosphorylation via a PI3K-mediated pathway. S1P-induced p38 kinase phosphorylation was also inhibited by LY294002 but not by U-73122 and BAPTA/AM (Fig. 5C), suggesting PI3K-dependency. PhS1P-induced ERK and p38 kinase phosphorylations were also inhibited by LY294002 but not by U-73122 and BAPTA/AM (Fig. 5D).

S1P and PhS1P induce chondrocyte proliferation via ERK-mediated pathway

In order to examine the effect of S1P on the regulation of cell proliferation, we investigated the effect of S1P on

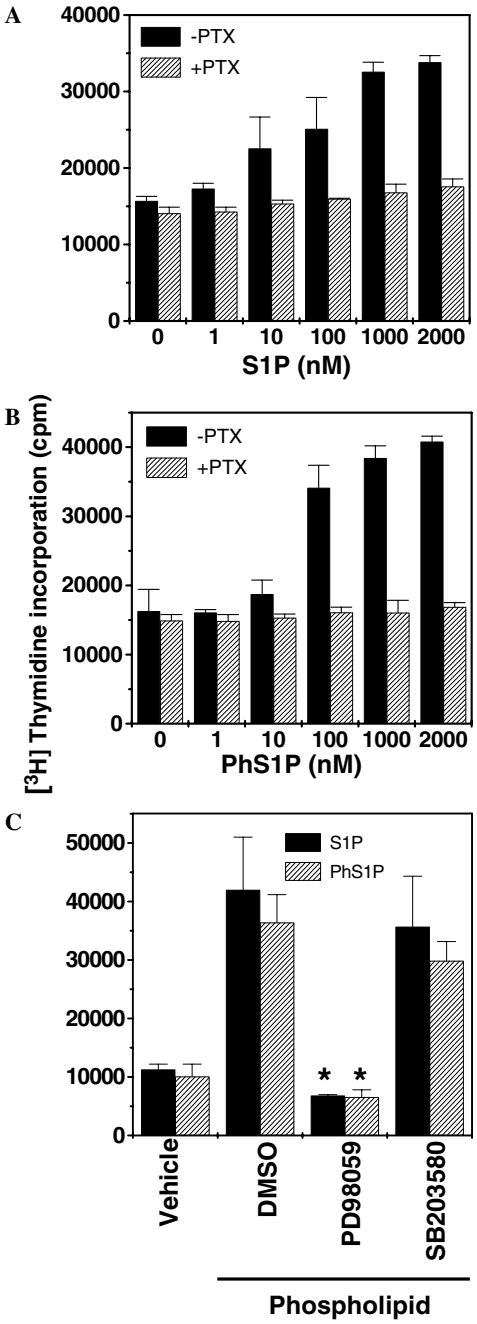

Fig. 6. Effect of S1P or PhS1P on chondrocyte proliferation. Rat primary chondrocytes (5×10^4) were treated with various concentrations of S1P (A) or PhS1P (B) for 24 h in the absence or presence of 100 ng/ml PTX. The cells were preincubated with vehicle, 50 μM PD98059 (60 min), or 20 μM SB203580 (15 min) prior to being treated with 2 μM S1P or 2 μM PhS1P for 24 h (C). Cells were harvested and [^{3}H]thymidine incorporation was measured. Vehicle is methanol. Results are presented as means ± SE of three independent experiments, which are performed in triplicate (A–C). * indicates results significantly different at the $p < 0.05$ probability levels as compared to the values obtained from the control (DMSO treated).

[^{3}H]thymidine incorporation in rat primary chondrocytes. As shown in Fig. 6A, the stimulation of rat primary chondrocytes by several concentrations of S1P for 24 h induced [^{3}H]thymidine incorporation in a concentration-dependent manner. The stimulation of rat primary chondrocytes with 2 μM S1P enhanced this [^{3}H]thymidine incorporation by around 2-fold versus the unstimulated control (Fig. 6A). PhS1P also induced [^{3}H]thymidine incorporation in a concentration-dependent manner. The stimulation of rat primary chondrocytes with 2 μM PhS1P enhanced this [^{3}H]thymidine incorporation by around 3-fold versus the unstimulated control (Fig. 6B). We, then, investigated the effect of PTX on S1P- or PhS1P-induced chondrocyte proliferation. When rat primary chondrocytes were preincubated with 100 ng/ml PTX prior to stimulation with S1P or PhS1P, the two phospholipids-induced [^{3}H]thymidine incorporations were almost completely inhibited (Fig. 6A and B), strongly suggesting the involvement of PTX-sensitive G proteins.

We also investigated the signaling pathways involved in the S1P-stimulated cell proliferation by using several enzyme-specific inhibitors. Fig. 6C shows that the S1P-induced [^{3}H]thymidine incorporation in rat primary chondrocytes was almost completely blocked in the presence of 50 μM PD98059, but not in the presence of 20 μM SB203580. PhS1P-induced [^{3}H]thymidine incorporation in rat primary chondrocytes was also almost blocked by 50 μM PD98059 but not by 20 μM SB203580 (Fig. 6C). These results suggest that ERK pathway is essential for [^{3}H]thymidine incorporation by S1P or PhS1P in rat primary chondrocytes.

Discussion

In this study, we demonstrated that S1P and PhS1P regulate cell proliferation in rat primary chondrocytes, suggesting that S1P receptor has a role in the modulation of arthritis. Since rat primary chondrocytes proliferation is important for the therapy of arthritis, these data on rat primary chondrocytes suggest that S1P receptor can be regarded as a crucial target for the therapy of arthritis. Previously we reported that LPA stimulates cell proliferation in rat primary chondrocytes [19]. In this study, we showed that S1P and PhS1P also stimulate chondrocyte proliferation. This finding strongly suggests not only proinflammatory cytokines, such as TNF-α and IL-1β, but also G-protein coupled receptor ligands like S1P and LPA might be involved in the regulation of chondrocyte function.

S1P binds to several isoforms of the cell surface receptors namely, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ [8,11–13]. When we performed RT-PCR to check the expression pattern of S1P receptors on rat primary chondrocytes, we found that rat primary chondrocytes express $S1P_2$, $S1P_3$, and $S1P_4$, but not $S1P_1$ (Fig. 1). In this study, we demonstrated that both of S1P and PhS1P stimulated cell proliferation in rat primary chondrocytes with similar potency (Fig. 6). Previous reports showed that PhS1P is a specific ligand for $S1P_4$ and $S1P_1$ [21,22]. Since rat primary chondrocytes do not express $S1P_1$ (Fig. 1), it suggests that PhS1P stimulates rat primary chondrocytes via $S1P_4$. According to previous reports, $S1P_1$ exclusively couples to G proteins of the $G_{i/o}$ family, whereas $S1P_2$, $S1P_3$, and $S1P_4$ are coupled to $G_{i/o}$ and also to G_q, G_s, and G_{13} [8,11–13,23]. In our study, we investigated the effect of PTX (which specifically inactivates $G_{i/o}$-mediated signaling pathways) on S1P- and PhS1P-induced signaling. When rat primary chondrocytes were pretreated with 100 ng/ml of PTX for 24 h prior to S1P or PhS1P stimulation, S1P- and PhS1P-induced cell proliferations were completely inhibited (Fig. 6A), and S1P- and PhS1P-stimulated ERK activations were also blocked by PTX pretreatment, as shown in Fig. 5A. These results suggest that S1P and PhS1P modulate ERK activation and lead to cell proliferation, and that PTX-sensitive G-protein coupled receptors are involved in these processes in rat primary chondrocytes.

To investigate the signal pathway of cell proliferation by S1P and PhS1P in rat primary chondrocytes, we checked the role of two different kinds of MAPKs. We observed that S1P stimulates both ERK and p38 kinase activity (Fig. 5A). To determine the role of ERK or p38 kinase on S1P- and PhS1P-induced cell proliferation, we pretreated the cells with two different MAPK-specific inhibitors, PD98059 and SB203580 (specific ERK and p38 kinase inhibitors, respectively). Preincubation of rat primary chondrocytes with PD98059 prior to S1P or PhS1P stimulation completely inhibited cell proliferation by S1P or PhS1P (Fig. 6C); however, SB203580 did not affect the two lipids-induced cell proliferations (Fig. 6C). This indicates that ERK, but not p38 kinase, plays a key role in S1P- or PhS1P-induced cell proliferations in rat primary chondrocytes. We also found that PI3K but not PLC-mediated Ca^{2+} plays a role in S1P-induced ERK activation in rat primary chondrocytes (Fig. 5B). In Fig. 3, we demonstrated that S1P stimulates $[Ca^{2+}]_i$ increase in a PTX-insensitive manner. Taken together the signaling pathways of S1P, it appears that S1P stimulates at least two different G-protein-coupled signalings; i.e., PLC-mediated $[Ca^{2+}]_i$ increase and MAPKs activation via PTX-sensitive G-protein-mediated pathways leading to cell proliferation in rat primary chondrocytes.

S1P levels in synovial fluid from osteoarthritis patients (~765 pM) have been reported to be much higher than levels in serum (~400 pM) or plasma (~100 pM) [24]. Furthermore, sphingosine kinase-1, which is essential for the generation of S1P from sphingosine, abundantly expressed in synovial tissue of osteoarthritis patients [25]. These results suggest the role of the enzymatic activity of sphingosine kinase-1 and the product (S1P) might be associated with arthritis pathogenesis. Further studies on the role of S1P on the functional regulation of chondrocyte activity are required. The present study indicates that S1P stimulates rat primary chondrocytes, resulting in cell proliferation via the S1P-specific receptors.

Acknowledgments

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