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Stimulatory Role of Lysophosphatidic Acid in Cyclooxygenase-2 Induction by Synovial Fluid of Patients with Rheumatoid Arthritis in Fibroblast-Like Synovial Cells

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While inflammatory cytokines are well-recognized critical factors for the induction of cyclooxygenase-2 (COX-2) in activated fibroblast-like synovial cells, the roles of biologically active components other than inflammatory cytokines in synovial fluid remain unknown. Herein, we assessed the role of lysophosphatidic acid (LPA), a pleiotropic lipid mediator, in COX-2 induction using synovial fluid of patients with rheumatoid arthritis (RA) in fibroblast-like RA synovial cells. Synovial fluid from RA patients stimulated COX-2 induction, which was associated with prostaglandin E2 production, in RA synovial cells. The synovial fluid-induced actions were inhibited by Giai protein inhibitor pertussis toxin and LPA receptor antagonist 3-(4-[4-(1-[2-chlorophenyl)ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid (Ki16425). In fact, LPA alone significantly induced COX-2 expression and enhanced IL-1α or IL-1β-induced enzyme expression in a manner sensitive to pertussis toxin and Ki16425. RA synovial cells abundantly expressed LPA1 receptor compared with other LPA receptor subtypes. Moreover, synovial fluid contains a significant amount of LPA, an LPA-synthesizing enzyme autotaxin, and its substrate lysophosphatidylcholine. In conclusion, LPA existing in synovial fluid plays a critical role in COX-2 induction in collaboration with inflammatory cytokines in RA synovial cells. Ki16425-sensitive LPA receptors may be therapeutic targets for RA. The Journal of Immunology, 2008, 181: 5111–5119.

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation in the synovium and a symmetric polyarthritis. Infiltrations of the synovial tissues by inflammatory cells such as macrophages and T cells occur, and local cellular proliferation of synoviocytes results in a marked expansion of the synovium called pannus, which invades and destroys articular structure (1, 2). Inflammatory mediators, including TNF-α and IL-1β, released from the inflammatory cells in response to various stimuli activate fibroblast-like synovial cells. Such synovial cells exhibit very unique characteristics in the process of bone resorption. Thus, synovial cells behave like osteoclasts in the induction of receptor activator of NF-κB ligand (RANKL), which is an essential ligand for the differentiation of bone-resorbing osteoclasts from their macrophage precursors (1, 3). Another important feature of synovial cells is that the cells stimulate cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) production, as do other inflammatory cells. The lipid mediator PGE2 is produced during inflammatory responses and is thought to be a major PG species working in RA pathogenesis, since a high level of PGE2 is detected in the synovial fluid of RA patients (4, 5) and PGE2 exhibits pleiotropic biological actions: for example, PGE2 mediates pain and inflammatory responses (1, 6, 7). Actually, COX-2 inhibitors are effective for decreasing pain in RA with less gastrointestinal side effects (6, 8), although some concerns of risk of cardiovascular events have recently been expressed (9, 10). In contrast, several studies suggest that PGE2 also mediates antiinflammatory effects as well by suppressing the production of proinflammatory cytokine and stimulating the synthesis of antiinflammatory cytokines (11, 12). Therefore, PGE2 is now regarded as a modulator rather than as a mediator of inflammatory responses.

It has been reported that a variety of cytokines, including TNF-α and IL-1β, are also present in synovial fluid of RA patients (4, 5) and are involved in COX-2 induction and PGE2 production (1, 6). The critical roles of the inflammatory cytokines in the progression of synovitis in RA are also evidenced from the observations that blocking Abs and antagonists against these cytokines are effective.

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4 Abbreviations used in this paper: RA, rheumatoid arthritis; RANKL, receptor activator of NF-κB ligand; COX-2, cyclooxygenase-2; IL, interleukin; PGE2, prostaglandin E2; Ki16425, 3-(4-[4-(1-[2-chlorophenyl)ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PTK, pertussis toxin; LPC, lysophosphatidylcholine; MG lipase, monoglyceride lipase; DGPP, dioctyl glycerol pyrophosphate.

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for the treatment of RA models in animals and RA patients (1). Thus, inflammatory cytokines are well-recognized critical factors for the induction of COX-2 in activated synovial cells. However, the roles of biologically active components other than inflammatory cytokines in synovial fluid remain unknown.

Lysophosphatidic acid (LPA), one of the simplest natural phospholipids, is a lipid mediator that evokes hormone- and growth factor-like responses in almost every cell type. Activating its G protein-coupled receptors, five of which have been identified so far (LPA1–LPA5), LPA elicits diverse cellular responses, including proliferation, survival, morphological change, and motility (13–15). LPA has been shown to be present in various biological fluids, including plasma (16), malignant ascites (17, 18), cerebrospinal fluid (19), and seminal fluid (20). In previous studies, however, to the best of our knowledge, no information was provided concerning the LPA actions and LPA receptor expression in synovial cells or the existence of LPA in synovial fluid. Only a few reports indicated the presence of soluble phospholipase A2, an LPA-synthesizing enzyme, in synovial fluid in patients with RA (21, 22).

In the present study, we first examined the effects of synovial fluid on COX-2 induction in fibroblast-like RA synovial cells and found a remarkable stimulation of COX-2 induction in association with PGE2 production. This activation was expected because of the presence of inflammatory cytokines; however, synovial fluid-induced actions were markedly inhibited by pertussis toxin (PTX), suggesting the involvement of G protein-coupled receptors in the synovial fluid-induced actions. This led to detailed investigations of the mechanisms underlying synovial fluid-induced COX-2 expression. We found that LPA, autotaxin, lysophospholipase D or an LPA-producing enzyme, and the enzyme substrate lysophosphatidylcholine (LPC) are present in synovial fluid and, moreover, that LPA enhanced inflammatory cytokine-induced COX-2 expression in synovial cells. The synovial fluid- and LPA-induced actions were markedly inhibited by 3-(4-[4-([1-(2-chlorophenyl) ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid (Ki16425), an antagonist for LPA receptors (LPA1 and LPA3) (23), suggesting a novel therapeutic target of LPA receptors in the treatment of RA.

Materials and Methods

Materials

1-Oleoyl-sn-glycero-3-phosphate (LPA) and sphingosine 1-phosphate (SIP) were purchased from Cayman Chemical; fatty acid-free BSA was from Calbiochem; PTX was from List Biological Laboratories; IL-1α and IL-1β were from BD Biosciences; mofezolac was from Mitsubishi Pharma; NS-398 was from Calbiochem; monoglyceride lipase (MG lipase) was from Asahi Kasei; and rabbit anti-actin Ab, dioctyl glycerol pyrophosphate (DGPP), and LPS from Escherichia coli 026:B6 were from Sigma-Aldrich. Ki16425 (23) was synthesized by Kirin Brewery. Rabbit anti-human COX-2 Ab was specifically isolated from antiserum obtained by immunizing animals with human COX-2 C-terminal peptide (ASSSRSGLD-DINPT) conjugated with keyhole limpet hemocyanin.

Preparation of RA synovial fluids

Synovial fluids were taken from the knees of six patients with RA by needle aspiration. All RA patients met the American Rheumatism Association criteria for the clarification of RA. Each sample was centrifuged at 3000 rpm for 30 min to remove possible inflammatory cells and blood cells and stored at −80°C. Informed consent was obtained from each patient for the use of samples, and the institutional medical ethics committee approved the study protocol.

Human fibroblast-like RA synovial cells

RA patients who fulfilled the American Rheumatism Association criteria for indication of synovectomy were selected, and informed consent was obtained from each patient for the use of synovial tissue. Synovial tissue was obtained from RA patient undergoing arthroplasty or synovectomy. The tissue specimens were minced into small pieces and treated with 5 mg/ml collagenase for 2 h at 37°C in serum-free Eagle’s MEM, filtered through a nylon mesh, and washed extensively. The cells were suspended in MEM supplemented with 20% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cultures were performed at 37°C in a humidified 5% CO2 atmosphere. The fibroblast-like cells adhered on culture dishes were harvested by trypsin treatment and used for experiments between the fourth and ninth passages (24).

Western blotting analysis of COX-2 expression in synovial cells

RA synovial cells (3 × 10⁴ cells) suspended in MEM containing 10% charcoal-treated FCS (MEM/CT-FCS) were seeded in a 60-mm tissue dish. After 24 h, the synovial cells were washed twice with serum-free media, starved with 0.5% FCS for 3 h, and then treated with synovial fluid for 24 h at 37°C. The cells were then harvested, and Western blot analysis was performed.

FIGURE 1. COX-2 protein expression induced by synovial fluid from RA patients in human RA synovial cells. A. Concentration-dependent COX-2 expression induced by synovial fluid. RA synovial cells were incubated for 6 h in the presence of indicated concentrations of synovial fluid B. The expression of COX-2 protein in the RA synovial cells was determined by Western blot analysis. The amounts of COX-2 were quantified by densitometer and normalized by the amounts of actin. Each column represents the mean ± SEM of three independent experiments. B. Effect of PTX on synovial fluid- or cytokine-induced COX-2 protein expression in RA synovial cells. RA synovial cells preincubated with or without 100 ng/ml PTX for 18 h were further incubated for 6 h with 10% synovial fluid B, 10 μg/ml IL-1α, or 10 ng/ml TNF-α. Representative results are shown.
culture plate and incubated at 37°C in a CO2 air incubator. After 42 h, nonadherent cells were removed and the adhered RA synovial cells were preincubated for 30 min in MEM/10% CT-FCS containing 1 μM Ki616425, 3 mM mofezolac, or 0.1 μM NS-398. Where indicated, PTX (100 ng/ml) was added to the culture medium 18 h before experiments. Subsequently, synovial fluid, LPA, S1P, or proinflammatory cytokine, such as IL-1 and TNF-α, was added and incubated at 37°C. The incubation was terminated by washing with ice-cold PBS followed by addition of 0.1 ml lysis buffer composed of 50 mM Tris (pH 7.8), 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholic acid, 0.01% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. The cells were then harvested from the dishes with a rubber policeman. The recovered lysate was settled on ice for 30 min and centrifuged at 14,000 × g for 30 min. The supernatant obtained was analyzed by Western blotting as follows: protein extracts were subjected to 7.5% SDS-PAGE, and proteins in the gel were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by electroblot. The membranes were blocked with 10 mM Tris-HCl (pH 8.0) for 1 h, and the blots were incubated with the primary Ab, which was raised against a peptide (DGLHDTEDKIKIQYC, 796–808 of human autotaxin) (19), was used. Other pro-taxin-specific polyclonal Ab, which was raised against a peptide (DGLHDTEDKIKIQYC, 796–808 of human autotaxin) (19), was used. Other anti-taxin-specific polyclonal Ab, which was raised against a peptide (DGLHDTEDKIKIQYC, 796–808 of human autotaxin) (19), was used. Other procedures for Western blotting were exactly the same as those described for COX-2 detection.

**RT-PCR analysis**

The mRNA levels for the subtypes of LPA receptors and S1P receptors were amplified by RT-PCR using specific primers for each receptor subtype (LPA1, sense 5'-ATCTTGGCAGATGGTCTGCA-3', and anti-sense 5'-TTGTGTGAACCTCCAGCCA-3'; LPA2, sense 5'-AAGCTGCACAGCCGCTGGCCCGG-3', and anti-sense 5'-dTGTCTGGCATGCGCACTT TGTTCC-3'; LPA3, sense 5'-AGGTCTAATGCACAGGC-3', and anti-sense 5'-GAGATGGTTGCAGAGGC-3'; LPA4, sense 5'-CTCCTGACTTCGACTACAC-3', and anti-sense 5'-GAGACGAGAGGAGGAGA-3'). For this purpose, total RNA was isolated from RA synovial cells using the RNeasy Kit (Qiagen). Reverse transcription was performed using reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The mRNA for COX-2 was amplified with the sense primer 5'-TTTCAATAG AGATTGTGGGAAAATTTGCT-3' and the anti-sense primer 5'-AGATCA TCTCTGCTGATGATCTT-3'. The mRNA for GAPDH was amplified with the sense primer 5'-AATTCATGGGACCCACGCGATG-3' and the anti-sense primer 5'-CATACACAGGGGGGACAGA-3', and used for normalization of the COX-2 mRNA expression level.

**Quantitative RT-PCR analysis**

To evaluate the expression level of mRNAs for LPA receptor subtypes (LPA1, LPA2, LPA3, and LPA4/GPR23), quantitative RT-PCR was performed using real-time TaqMan technology with a sequence detection system (model 7700, Applied Biosystems) described previously (25). The specific probes for LPA receptors were obtained from TaqMan gene expression assays (Applied Biosystems; ID numbers of the products are Hs00173500 for LPA1, Hs00173704 for LPA2, Hs00173857 for LPA3, and Hs00173926 for LPA4). The mRNA levels of the targeted mRNAs were normalized to the relative ratio of the expression of GAPDH mRNA.
Measurement of endotoxin levels in test samples

To exclude the possibility of endotoxin contamination, endotoxin levels in the assay medium and the test samples were determined using an endotoxin assay reagent, Endospecy, according to the manufacturer’s instruction (Seikagaku). The concentration of endotoxin was expressed as EU/ml. Any significant amount of biologically active endotoxin was not detected in the test samples, including 10^9/mL H9262 M LPA, 10 pg/ml IL-1, 10% synovial fluids and assay medium: all samples analyzed were out of range by the endotoxin assay method used, and the endotoxin concentration, even if present, was calculated to be 0.0015 EU/ml, which corresponds to 0.003 pg/ml LPS.

Evaluation of contents of S1P, LPA, and LPC in synovial fluid

S1P and LPA were selectively extracted as alkaline-soluble lipids as described previously (26). By this procedure, major lipid components, such as phosphatidylcholine, sphingomyelin, and other neutral lipids, can be removed. The S1P content was evaluated based on the ability of S1P to displace labeled S1P on S1P1 receptor (26) or to stimulate S1P3 receptor-mediated inositol phosphate production as described previously (27). Evaluation of LPA-equivalent activity was performed by a sensitive and specific bioassay based on the ability of LPA to inhibit cAMP accumulation in LPA1-expressing RH7777 cells as described previously (18). The LPA-equivalent activity in the test sample was expressed as an LPA C18:1-equivalent level. Please note that this bioassay is unsuitable for a quantitative measurement of LPA; however, it excludes LPA species that cannot stimulate LPA1 receptors. Thus, the bioassay is superior to know “active” LPA-equivalent content to stimulate LPA1 receptors. To measure LPC content, synovial fluid (0.2 ml) was extensively mixed with chloroform (1 ml), methanol (1 ml), 1 M KCl (0.6 ml), and 1 N HCl (0.05 ml), and the phases were separated. LPC was then separated by an HPTLC using a solvent system consisting of chloroform, methanol, and 20% NH₄OH (60/35/8). The bands were stained with primulin and visualized under UV light as described previously (19). The content of LPC was evaluated from the density of standard LPC spot.

FIGURE 3. Inhibitory effect of antagonist (Ki16425) for LPA receptor on the LPA- and synovial fluid-induced COX-2 expression in RA synovial cells. A. Dose-dependent inhibitory effect of Ki16425 on the LPA-induced COX-2 expression. RA synovial cells were preincubated with the indicated concentrations of Ki16425 for 30 min and further incubated with or without 10 μM LPA for 5 h. Data represent the means ± SEM of three independent experiments. **, Effect of Ki16425 was significant (p < 0.01). B. Effect of Ki16425 on synovial fluid-induced COX-2 protein expression in RA synovial cells. RA synovial cells were incubated for 5 h with 10 μM LPA, 10 μM S1P, or 10% synovial fluids (B, C, and R6) from three RA patients in the presence or absence of 1 μM Ki16425. Representative results are shown. C. Effects of PTX and Ki16425 on LPA- or synovial fluid-induced COX-2 mRNA expression in RA synovial cells. RA synovial cells were incubated for 4 h with 10 μM LPA or 10% synovial fluid B in the presence or absence of 1 μM Ki16425. For PTX treatment, the cells were pretreated with 100 ng/ml PTX (18 h). Amounts of COX-2 mRNA were quantified by real-time PCR. Each column represents the mean ± SEM of three independent experiments.

FIGURE 4. Effects of MG lipase, an LPA-degrading enzyme, on COX-2 induction. LPA (50 μM), synovial fluid C or R6 (50%), or IL-1α (50 pg/ml) was treated with or without MG lipase at 10 U/ml for 30 min at 37°C in MEM containing 0.1% BSA. The agents treated with (+) or without (−) MG lipase were then added to the incubation medium of synovial cells at a final concentration of 20% of the initial concentration. The cells were incubated for 5 h to analyze COX-2 expression by Western blot.
Data presentation

The results of multiple observations are presented as the means ± SEM of three independent experiments unless otherwise stated. Statistical significance was assessed by the Student’s t test.

Results

RA synovial fluids induced COX-2 expression in RA synovial cells in a manner sensitive to PTX

As shown in Fig. 1A, the synovial fluid of an RA patient (synovial fluid B) at concentrations of 1–10% clearly induced COX-2 protein expression. This induction of the enzyme by synovial fluid was expected because synovial fluid is known to contain a variety of cytokines that induce COX-2 expression (4, 5). In fact, IL-1α stimulated COX-2 expression, although we did not observe a significant effect on the enzyme expression by TNF-α in our system (Fig. 1B). However, the synovial fluid-induced COX-2 expression was markedly inhibited by PTX, whereas IL-1α-induced enzyme expression was hardly affected by the toxin treatment (Fig. 1B).

These results suggest that PTX-sensitive G protein-coupled receptors are involved in synovial fluid-induced COX-2 expression.

Induction of COX-2 expression by LPA and S1P

We examined the possible components involved in the synovial fluid-stimulated enzyme induction. Consistent with a previous report (28), a lipid mediator, S1P, stimulated COX-2 expression in a time- (Fig. 2A) and dose- (Fig. 2B) dependent manner. We also examined the effect of another lipid mediator, LPA, on the enzyme expression and found that LPA is as effective as S1P to induce COX-2 expression (Fig. 2, A and B). The effects of PTX on the S1P- and LPA-induced actions are shown in Fig. 2C. The LPA effect was markedly inhibited by PTX, and the S1P effect was weakly inhibited by the toxin.

FIGURE 5. RA synovial fluid- or LPA-induced PGE2 generation in RA synovial cells. A, Time-dependent PGE2 generation induced by LPA. RA synovial cells were incubated with or without 3 μM LPA. The amounts of PGE2 released into the supernatant were determined by ELISA. Data represent the means ± SEM of the data obtained with the representative experiment done in triplicate. B, Concentration-dependent PGE2 generation induced by synovial fluid or LPA. RA synovial cells were incubated for 8 h with the indicated concentrations of LPA or synovial fluid B. Without synovial cells, PGE2 was not detected under the experimental condition. Data represent the means ± SEM of the data obtained with the representative experiment done in triplicate. C, Effects of PTX, Ki16425, or COX inhibitors on 3 μM LPA-induced PGE2 generation in RA synovial cells. RA synovial cells were incubated for 8 h with or without 3 μM LPA in the presence or absence of 1 μM Ki16425, 3 nM mofezolac, or 0.1 μM NS-398. As for PTX treatment, the cells were pretreated with 100 ng/ml PTX for 18 h. Each column represents the mean ± SEM of three independent experiments.

FIGURE 6. Expression of mRNA for LPA receptors and S1P receptors in RA synovial cells. A, Expressions of mRNA for LPA receptors and S1P receptors were detected by RT-PCR as described in Materials and Methods. B, The amounts of mRNA for LPA receptors quantified by TaqMan real-time PCR method. Each column represents the mean ± SEM of three independent experiments. C, Effects of Ki16425 and DGPP on the LPA-induced COX-2 expression. RA synovial cells were preincubated for 30 min in the presence or absence of 1 μM Ki16425 or 3 μM DGPP and further incubated with 10 μM LPA for 5 h. The expression of COX-2 protein in the synovial cells was analyzed by Western blot. Representative results are shown.
Involvement of LPA in synovial fluid-stimulated COX-2 expression and PGE₂ production

We then examined the effects of Ki16425, an LPA receptor antagonist, on LPA- and synovial fluid-stimulated COX-2 expression. Ki16425 dose-dependently inhibited LPA-stimulated COX-2 expression (Fig. 3A). The expressions of COX-2 protein induced by synovial fluids from three RA patients were also markedly inhibited by Ki16425 for 30 min and then incubated for 6 h with L-1α in the presence or absence of 10 μM LPA. Each column represents the mean ± SEM of three independent experiments. C. Effect of Ki16425 on synergism of IL-1α and LPA in COX-2 protein expression. RA synovial cells were pretreated with 1 μM Ki16425 for 30 min and then incubated for 6 h with IL-1α in the presence or absence of 10 μM LPA. The amounts of PGE₂ released into the supernatant were determined by ELISA. Each column represents the mean ± SEM of the representative experiment done in triplicate. D. Effect of Ki16425 on synergism of IL-1α and LPA in PGE₂ generation. RA synovial cells were incubated for 6 h with LPS at 0, 0.1, or 1.0 pg/ml in the presence or absence of 10 μM LPA, or with 10% synovial fluid (R6). The expression of COX-2 protein in the RA synovial cells was determined by Western blot analysis.
Possible involvement of LPA₁ in LPA-induced COX-2 expression

To examine LPA receptor subtypes possibly involved in LPA-induced COX-2 expression in RA synovial cells, we analyzed the mRNA expression of LPA₁, LPA₂, and LPA₃ by RT-PCR and real-time RT-PCR. The mRNA expressions of S1P₁, S1P₂, and S1P₃ were also analyzed by RT-PCR. As shown in Fig. 6, RA synovial cells expressed several types of S1P receptor mRNA, including S1P₁, S1P₂, and S1P₃ (Fig. 6A). In addition, LPA₁ mRNA expression, but not those of LPA₂ and LPA₃ mRNA, was detected (Fig. 6A and B). Moreover, LPA-induced COX-2 expression was susceptible to an LPA₁ and LPA₃-specific antagonist, Ki16425, but not to an LPA₂-specific antagonist, DGPP (Fig. 6C). These results imply the possible involvement of LPA₁ in the LPA-induced COX-2 expression. To confirm the involvement of LPA₁ receptors, we employed small interfering RNA specific to LPA₁ receptors to knock down the receptors. Although we succeeded with >80% reduction in LPA₁ mRNA expression by the small interfering RNA, we failed to detect a significant change in LPA₁- and synovial fluid-induced COX-2 expression (data not shown).

Enhancement of cytokine-induced COX-2 expression and PGE₂ production by LPA

The foregoing results suggested the involvement of LPA in synovial fluid-induced actions. However, the maximal effects of LPA on COX-2 induction and PGE₂ production were almost the same as those shown in Fig. 3C or sometimes less than those by synovial fluid (Figs. 5B and 5B), suggesting the participation of another regulatory mechanism in the synovial fluid-induced actions. In synovial fluid, a variety of cytokines, including TNF-α, IL-1-α, and IL-1β, have been shown to be present and involved in COX-2 induction and PGE₂ production in synovial cells (4, 5, 29). As shown in Fig. 7A, IL-1α at 10 pg/ml stimulated COX-2 induction more effectively than did a maximal concentration of LPA at 10 μM. Even though the LPA effect alone was small, the lipid mediator synergistically enhanced the IL-1α- and IL-1β-induced COX-2 expression (Fig. 7A and B). The enhancement of the IL-1 action by LPA on COX-2 expression was markedly inhibited by Ki16425 (Fig. 7C). We also examined the LPA action on PGE₂ production and found that LPA at 3 μM stimulated net PGE₂ production from ~0.08 to 0.1 ng/3 × 10⁴ cells in the presence of IL-1α (Fig. 5B and C) to ~2.5 ng/3 × 10⁴ cells in its presence (Fig. 7D). As expected, the LPA effect was completely inhibited by Ki16425 (Fig. 7D). Similar enhancement of COX-2 induction and PGE₂ production in response to IL-1α was also observed by the treatment of the cells with S1P instead of LPA (data not shown).

As shown in Fig. 7E, the exogenous LPS also induced COX-2 expression, and its expression was enhanced by LPA. The minimal concentration of LPS required for the significant induction of COX-2 expression by itself and the synergistic enhancement of the expression by LPA was 0.1–1 pg/ml. This result implies that if synovial fluid contains endotoxin >0.1 pg/ml, the activity of synovial fluid to induce COX-2 expression might be explained by the contaminated endotoxin. We therefore verified the amount of endotoxin. However, no significant amount of biologically active endotoxin was detected in the samples including 10 μM LPA, 10 pg/ml IL-1, 10% synovial fluids, and assay medium: all samples analyzed were out of range by the endotoxin assay method, and its concentration was calculated to be <0.0015 EU/ml. On the other hand, the biologically active endotoxin concentration in LPS at 0.1 pg/ml was estimated to be 0.0489 ± 0.0034 EU/ml. Thus, it is unlikely that the contaminated endotoxin is involved in COX-2
induction by various stimuli including IL-1, LPA, and synovial fluids in our assay system.

Detection of LPA, LPC, and LPA-producing enzyme autotaxin in synovial fluid

To confirm the role of LPA in synovial fluid-induced COX-2 induction, we evaluated the LPA content in synovial fluid: LPA C18:1-equivalent level in 10% synovial fluid from patients with RA was estimated to be 0.37 ± 0.22 μM as an average, which corresponds to 3.7 ± 2.2 μM in the original synovial fluid (Fig. 8A). In contrast, no significant amount of S1P was detected in RA synovial fluid: all six samples were out of range by our assay method, and the content was calculated to be <3 nM (data not shown).

The LPA level in the synovial fluid was just the threshold to enhance the cytokine-induced action when synovial fluid was used at 10%; a minimal effective dose of LPA to enhance the cytokine-induced COX-2 expression was 0.1–0.3 μM (Fig. 7A). However, we observed and ~4.2-fold (as an average) increase in LPA during a 4-h incubation of 10% synovial fluid in the culture medium (Fig. 8A). These results suggest that synovial fluid possesses LPA-producing activity. Actually, we found autotaxin, lysophospholipase D, or an LPA-synthesizing enzyme (30) (Fig. 8B) and its substrate LPC (Fig. 8C) in all RA synovial fluid samples employed, although there seems to be no clear correlation between LPA level and either autotaxin expression or LPC level (Fig. 8). The LPC concentration was roughly estimated from the standard sample of LPC (160) to be 30–250 μM in synovial fluid.

Discussion

In the present study, we showed that LPA in synovial fluid plays an important role in the stimulation of COX-2 expression and PGE2 production in RA synovial cells. The synergistic induction of COX-2 by LPA and cytokines, such as IL-1α and IL-1β, may explain the strong stimulation of enzyme induction and PGE2 production by synovial fluid from RA patients. We further showed that an LPA receptor antagonist, Ki16425, markedly inhibited these synovial fluid-induced actions. The concentration of IL-1β in synovial fluid has been reported to be as high as 10–40 pg/ml (4, 5). Although LPA at 10 μM showed a rather small effect on COX-2 induction, the cytokine effect was remarkably enhanced by LPA. Thus, the dose-response curve of IL-1β on COX-2 induction was roughly shifted one order to the left in the presence of LPA. As a result, IL-1β at 1 pg/ml, of which concentration is supposed to be present in 10% synovial fluid employed in the present study, only slightly stimulated COX-2 induction in the absence of LPA but was clearly augmented by its presence.

LPA also enhanced IL-1α-induced COX-2 expression. In synovial fluid, we observed the presence of ~3.7 μM LPA as an LPA C18:1-equivalent level. This concentration of LPA, however, is just the threshold to explain the participation of LPA in the synovial fluid-induced action because 370 nM LPA, which is assumed to be present in 10% synovial fluid, is expected to significantly enhance the cytokine-induced COX-2 expression (Fig. 7). Moreover, we detected an LPA-producing activity in synovial fluid. This activity may be partly explained by the presence of autotaxin, an LPA-synthesizing enzyme, and its substrate LPC in the synovial fluid, although the LPA-producing activity of the synovial fluid does not seem to be strictly correlated with the autotaxin expression level and/or LPC content. Another type of LPA-synthesizing enzyme, such as a soluble phospholipase A2, might also play a role in LPA synthesis in RA synovial fluid (21, 22). In any event, the synergistic enhancement of COX-2 expression by LPA and cytokines may explain the high activity of synovial fluid to stimulate the enzyme induction, although either cytokines or LPA alone at concentrations existing in synovial fluid might be unable to exert the high activity obtained by synovial fluid.

In a recent study (28), S1P was shown to enhance TNF-α- and IL-1β-induced COX-2 expression. In contrast to the previous study (28), however, we failed to detect a significant amount of S1P in the synovial fluid by our assay method. Thus, all six samples employed were out of range by our assay method, and the content was calculated to be <3 nM. At present, the discrepancy of the results between the present study and the previous one (28) remains unknown. However, the involvement of S1P in the synovial fluid-stimulated COX-2 induction, even if not negligible, may be small. The synovial fluid- and LPA-induced action was markedly inhibited by PTX and Ki16425, while the S1P-induced action was weakly inhibited by PTX and was never inhibited by Ki16425.

The LPA actions on COX-2 induction, regardless of the presence of cytokines, were susceptible to Ki16425, an LPA receptor antagonist, and PTX, a Gαi/o protein inhibitor. Among LPA receptor subtypes 1–5, LPA1 and LPA3 are particularly sensitive to Ki16425 (23). The present study showed that RA synovial cells expressed LPA1 mRNA but not LPA2 and LPA3 mRNA. Moreover, the LPA3-specific antagonist DGPP failed to inhibit the LPA action. These results suggest that the Gαi/o protein-coupled LPA1 receptor may be involved in the LPA- and, hence, synovial fluid-induced actions. Although we failed to confirm the involvement of LPA1 receptor by the experiments using LPA1-specific small interfering RNA, this does not exclude the possible involvement of LPA1 receptors in the LPA-induced actions because LPA1 receptor expression may be high, as estimated from the result of the ratio of LPA1 vs GAPDH of 0.035. Additional experiments are necessary to identify the LPA receptor subtypes involved in the synovial cell regulation and the sources of LPA in synovial fluid.

Although treatment of RA patients with TNF-α blockers has been shown to improve the inflammatory responses in RA, these drugs do not induce complete remission (31). Such limited therapeutic effects of TNF-α can be explained by the involvement of cytokines, such as IL-1, other than TNF-α in RA progression (32). Thus, the development of therapeutic means targeted on COX-2 induction and PGE2 production in synovial cells is still an important aspect in the prevention of painful synovitis in RA patients. In addition to cytokines, the present study suggested a critical role of LPA in inflammatory responses in RA. Ki16425 has recently been shown to inhibit the migration and proliferation of cancer cells (18, 33–35) and smooth muscle cells (36) in vitro and of bone metastasis of ovarian and breast cancer cells in vivo (35). Thus, Ki16425 has been suggested to have potential as a therapeutic drug for cancer and vascular diseases. The present study further suggested that Ki16425 has potential as a drug for RA. After this work was in the review process, a paper appeared describing that RA synovial fluid contains autotaxin and that LPA receptors are involved in the synovial fluid-induced cell migration and production of cytokines, including IL-8 and IL-6 (37). This report is compatible with our results with respect to the presence of autotaxin in the synovial fluid.

In conclusion, LPA in synovial fluid in RA patients plays a stimulatory role in COX-2 induction and PGE2 production in collaboration with cytokines. Autotaxin and LPC in synovial fluid may at least partly function as a system to supply LPA. LPA-induced COX-2 expression is mediated through Gαi/o-coupled and Ki16425-susceptible LPA receptor, possibly LPA1, although the involvement of LPA3 is still possible. LPA-producing systems and LPA receptors may be novel therapeutic targets for painful RA.
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Disclosures

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