Identification of lysophospholipid receptors in human platelets: the relation of two agonists, lysophosphatidic acid and sphingosine 1-phosphate

Ken Motohashi[a], Satomi Shibata[a], Yukio Ozaki[b], Yutaka Yatomi[b], Yasuyuki Igarashi[a,*]

[a] *Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12-Nishi-6, Kita-ku, Sapporo 060-0812, Japan*

[b] *Department of Laboratory Medicine, Yamanashi Medical University, Nakakoma, Yamanashi 409-3898, Japan*

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Abstract Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (Sph-1-P) are known as structurally related bio-active lipids activating platelets through their respective receptors. Although the receptors for LPA and Sph-1-P have been recently identified in various cells, the identification and characterization of ones in platelets have been reported only preliminarily. In this report, we first investigated the distinct modes of LPA and Sph-1-P actions in platelet activation and found that LPA functioned as a much stronger agonist than Sph-1-P, and high concentrations of Sph-1-P specifically desensitized LPA-induced intracellular Ca^{2+} mobilization. In order to identify the responsible receptors underlying these observations, we analyzed the LPA and Sph-1-P receptors which might be expressed in human platelets, by RT-PCR. We found for the first time that Edg2, 4, 6 and 7 mRNA are expressed in human platelets.

Key words: Edg family; G-protein-coupled receptor; Lysophosphatidic acid; Platelet; PSP24; Sphingosine 1-phosphate

1. Introduction

Sphingosine 1-phosphate (Sph-1-P), one of the sphingolipid metabolites, has been demonstrated to function as a lipid mediator which is involved in cell motility [1,2], mitogenesis [3,4] and cell shape changes including neurite retraction [5] and regulation of angiogenesis [6]. Recent studies have suggested that Sph-1-P acts both intracellularly and extracellularly to cause various biological responses [7–9].

In platelets, Sph-1-P is abundantly stored intracellularly [10], and released upon stimulation with thrombin or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [10,11]. Sph-1-P functions as an extracellular mediator for aggregation and intracellular Ca^{2+} mobilization in platelets, although weakly [10,11]. In these studies, however, the characterization of receptor molecule(s) for this lipid has not been challenged [10]. Very recently, receptors for lysophosphatidic acid (LPA) and Sph-1-P have been identified in various cells [12–18]; the Edg family, which are the class of seven transmembrane G protein-coupled receptors for lysophospholipids, comprises at least seven members, Edg1–Edg7 [17–19]. Specific receptors for Sph-1-P are Edg1, 3, 5 [20–25] and those for LPA are Edg2, 4, 7 [13,15,18,26]. Although Edg1 is a high affinity receptor for Sph-1-P, it has been demonstrated that LPA also acts on Edg1 with a low affinity [27]. In contrast, the LPA receptor Edg2 has been suggested to function as a possible low affinity receptor for Sph-1-P in human osteosarcoma MG63 cells [28]. Although a ligand for Edg6 has not been clearly identified yet, it is postulated to be a Sph-1-P receptor from the structural resemblance with the Sph-1-P receptors so far identified [17]. In addition, the presence of another class of G protein-coupled receptor for LPA, named PSP24, was discovered [29,30].

In this work, we investigated the distinction and relation of modes of action of LPA and Sph-1-P in human platelet activation and examined the expression of the corresponding receptors in human platelets.

2. Materials and methods

2.1. Materials

The following materials were obtained from the indicated suppliers: Sph-1-P (Biomol, Plymouth Meeting, PA, USA); LPA (Sigma, St. Louis, MO, USA); thrombin (Mochida Pharmaceutical Co., Tokyo, Japan); ionomycin (Calbiochem, San Diego, CA, USA); fura2-AM (Dojindo Laboratories, Kumamoto, Japan).

2.2. Platelet preparation

Washed human platelets for intracellular Ca^{2+} mobilization and platelet aggregation were prepared as described previously [31] and finally suspended in a buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$, 1 mg/ml glucose, and 20 mM HEPES (pH 7.4), at a cell density of 3×10^8/ml. When platelet aggregation was measured, the suspensions were supplemented with 1 mM $CaCl_2$ and 500 μg/ml of fibrinogen. When platelet intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement was performed, the suspensions were supplemented with 1 mM $CaCl_2$ or EGTA, as indicated. All experiments using intact platelet suspensions were performed at 37°C.

Human platelets for mRNA isolation were prepared as follows. 200 ml of blood containing 15% of acid citrate dextrose solution (0.8% citric acid, 2.2% trisodium citrate, 2.45% glucose) was collected from healthy adult volunteers. The blood samples were centrifuged at 1000 rpm for 20 min at room temperature. Platelet-rich plasma, obtained from the upper one-third of the blood sample, was transferred to a new tube for another centrifugation (900 rpm for 2 min) to avoid contamination with leukocytes. The platelet-rich plasma was then transferred to another tube and mixed with an equal volume of phosphate buffered saline (PBS) containing 15% acid citrate dextrose solution, followed by 1 μM prostaglandin E_1 addition. The sample was

*Corresponding author. Fax: (81)-11-706 4986.
E-mail: yigarash@pharm.hokudai.ac.jp

Abbreviations: LPA, lysophosphatidic acid; PCR, polymerase chain reaction; PMN, polymorphonuclear; RT, reverse transcriptase; Sph-1-P, sphingosine 1-phosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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passed through Sepacell PLX-5A-ILS (Asahi Medical Co., Ltd.); the column was used for exclusion of leukocytes, and the flow-through sample was centrifuged (3000 rpm for 10 min at room temperature), and the resultant plasma supernatant was carefully removed from the platelet pellet. The pellet was washed with PBS containing 15% acid citrate dextrose solution and 1 μM prostaglandin E_1. For successful analysis in applying RT-PCR, it was crucial to confirm that platelet preparations contained no contaminating cells. To estimate contamination in the suspension, a hemocytometer was used for cell counting. We confirmed that platelets were more than 99.99% pure, by estimating the ratio of white blood cells, if any, to platelets.

2.3. Preparation of polymorphonuclear leukocytes

For preparation of polymorphonuclear (PMN) leukocytes, 50 ml of blood treated with heparin (as an anticoagulant) was collected from healthy adult volunteers. The sample was carefully layered over an equal volume of Polymorphprep™ (Nycomed Pharma AS, Oslo, Norway), and centrifuged at 500×*g* for 30 min at 20°C. The lower band, formed after centrifugation, consisted of PMN leukocytes. The cells were diluted by addition of 0.45% NaCl and centrifuged.

2.4. Cell culture

Jurkat is a human T-lymphocyte cell line from an acute T-cell leukemic patient. Jurkat cells were obtained from the American Type Culture Collection and were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.5. Platelet aggregation

This process was determined in a Platelet Ionized Calcium Aggregometer (Chrono-Log, Havertown, PA, USA) under continuous stirring at 1000 rpm. The instrument was calibrated with a platelet suspension for zero light transmission and the buffer for 100% transmission. The increase in light transmission caused by platelet aggregation was continuously recorded. For quantitative evaluation of platelet aggregation, results were expressed as the maximum change of light transmission within 5 min after stimulation.

2.6. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ measurement was performed with the use of the Ca^{2+}-sensitive fluorophore fura2 as described previously [32] except that the fluorescence measurements were made using a FS100 (Kowa, Tokyo, Japan). The $[Ca^{2+}]_i$ values were determined from the ratio of fura2 fluorescence intensity at 340 and 380 nm excitation.

2.7. Isolation and RT-PCR of mRNAs from platelets, PMN leukocytes and Jurkat cells

The mRNAs of platelets, PMN leukocytes and Jurkat cells, prepared as described above, were isolated by QuickPrep Micro mRNA Purification Kit (Pharmacia). Aliquots of mRNA preparation were frozen at −80°C until use. Based upon the nucleotide sequences of the human Edg family (Edg1–7), PSP24 and platelet factor 4 (PF4) in the database, oligonucleotide primers were prepared (Table 1). RT-PCR was carried out in a Thermal Cycler (GeneAmp PCR system 9700, PE Applied Biosystems) using the SuperScript™ One-Step™ RT-PCR System (Life Technologies) with the template mRNA (50 ng) under the following conditions: (1) 50°C for 30 min; (2) 94°C for 2 min; (3) 94°C for 15 s; (4) 55°C for 30 s; (5) 72°C 30 s; (6) repeat of steps 3–5 for 40 cycles; and (7) 72°C for 1 min. When EDG6 primers were used for RT-PCR, the annealing temperature was changed from 55°C to 58°C at step 4. RT-PCR products were visualized on 2% agarose gel electrophoresis, and their nucleotide sequences were confirmed with a fluorescent DNA sequencer (ABI-377, PE Applied Biosystems).

3. Results

3.1. Distinct modes of platelet aggregation and intracellular Ca^{2+} mobilization induced by Sph-1-P and LPA

LPA is a potent platelet agonist, while Sph-1-P is a weak one. As shown in Fig. 1, 10 μM LPA induced a strong and irreversible platelet aggregation; Sph-1-P, at a concentration as high as 40 μM, induced only a weak and reversible aggregation. When platelet intracellular Ca^{2+} mobilization was measured in the presence of extracellular Ca^{2+}, LPA induced a marked increase in intracellular Ca^{2+} concentrations in a dose-dependent manner (Fig. 2). The response induced by Sph-1-P was much weaker, as was the case with platelet aggregation (Fig. 2). When platelets were suspended in Ca^{2+}-free buffer, which was further supplemented with EGTA (final 1 mM), similar results were obtained. However, maximum Ca^{2+} mobilization was obtained with 10–20 μM for LPA; above these concentrations, the LPA-induced response weakened for unknown reason(s) (data not shown). Accordingly, LPA functions as a strong platelet agonist, but Sph-1-P functions as a weak one in terms of aggregation and intracellular Ca^{2+} mobilization.

3.2. Desensitization of LPA-induced platelet intracellular Ca^{2+} mobilization by Sph-1-P

It was shown that LPA and Sph-1-P function as an extracellular mediator for platelet activation [11,33–35], Furthermore, it has been suggested that LPA and Sph-1-P may share membrane surface receptor(s) because they have common structural features [10,36]. We performed desensitization experiments with these two lipids for the intracellular Ca^{2+} mobilization in platelets. LPA (5 μM) induced irreversible aggregation and increase in intracellular Ca^{2+} concentrations (Figs. 1B and 2). The pretreatment with Sph-1-P (40 μM) inhibited the LPA (5 μM)-induced intracellular Ca^{2+} mobilization in platelets (Fig. 3). In contrast, Sph-1-P (40 μM) did not affect the response induced by ionomycin (50 nM) or thrombin (0.04 U/ml) (Fig. 3). The specific desensitization of LPA-induced Ca^{2+} mobilization by Sph-1-P supported again the idea that LPA and Sph-1-P may share cell surface receptor(s) in human platelets.

3.3. Expression of lysophospholipid receptors for human platelets

In order to search the expression of lysophospholipid receptors in human platelets, we investigated mRNA expression

Table 1
Primers of RT-PCR for Edg family and PSP24

Edg family	Forward primer	Reverse primer
Edg1	5′-cctcttcctgctaatcagcg-3′	5′-acaggtcttcaccttgcagc-3;′
Edg2	5′-cggagactgactgtcagcac-3′	5′-ggtccagaactatgccgaga-3′
Edg3	5′-tcagcctgtctcccacggtc-3′	5′-acggctgctggacttcacca-3′
Edg4	5′-cccaaccaacaggactgact-3′	5′-gagcccttatctctccccac-3′
Edg5	5′-cattgccaaggtcaagctgt-3′	5′-acgatggtgaccgtcttgag-3′
Edg6	5′-acgggagggcctgctcttca-3′	5′-aaggccagcaggatcatcag-3′
Edg7	5′-ggacacccatgaagctaatg-3′	5′-tctgggttctcctgagagaa-3′
PSP24	5′-gtggacttgagcttcaagac-3′	5′-cactttggggaggatttgga-3′

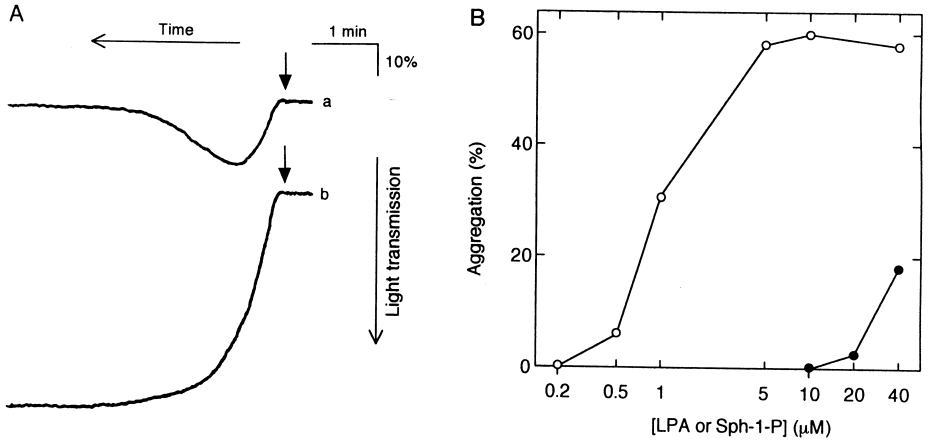

Fig. 1. A: Platelet aggregation induced by LPA or Sph-1-P. Human platelets were challenged with 10 μM LPA (b) or 40 μM Sph-1-P (a), as indicated by arrows. B: Platelet aggregation induced by various concentrations of LPA (open circles) or Sph-1-P (closed circles) is shown. Results are expressed as means of two typical determinations.

of Edg family and PSP24 receptors by RT-PCR, since Northern blot analysis seemed practically impossible for platelets, which contain a very small amount of mRNA. The mRNAs of Edg1-7 and PSP24 are all detected in Jurkat human T-lymphocytes, which were hence used as positive controls to confirm that the prepared primers worked properly (Table 1 and Fig. 4A, lanes 1–8). Furthermore, mRNA of platelet factor 4 (PF4), which is known to be specifically expressed in platelets, was amplified from human platelet mRNA preparation (Fig. 4B, lane 9), confirming that our preparation of platelet mRNA was successful. When the platelet mRNA preparation was used as a template for RT-PCR, the mRNAs of Edg2, 4, 6 and 7 were clearly detected (Fig. 4B, lanes 2, 4, 6 and 7). When the PMN leukocyte mRNA preparation was used, mRNAs of Edg1-6 and PSP24 were detected (Fig. 4C, lanes 1–8). The absence of amplification of the Edg1, Edg3, Edg5 and PSP24 mRNAs in platelets, which were clearly expressed in leukocytes, further excluded the possibility of leukocyte mRNA contamination in the platelet mRNA preparation (Fig. 4B,C).

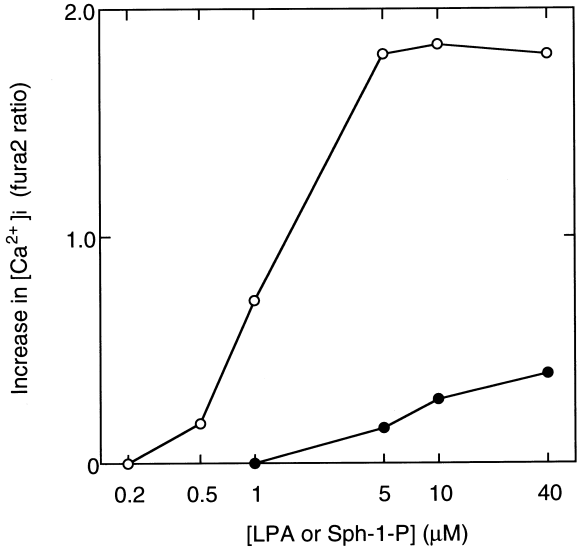

Fig. 2. Platelet intracellular Ca^{2+} mobilization induced by LPA or Sph-1-P. Fura2-loaded platelets supplemented with 1 mM $CaCl_2$ were challenged with various concentrations of LPA (open circles) or Sph-1-P (closed circles). The results are the values of peak increases after stimulation and are from a single experiment representative of three. In separate experiments, the basal $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by 40 μM Sph-1-P were found to be 88 ± 11 nM and 128 ± 12 nM (mean ± S.D., $n = 4$), respectively.

4. Discussion

LPA and Sph-1-P are a newly described family of lipid mediators, inducing human platelet aggregation [11,34,36]. In our previous work, we observed that Sph-1-P desensitizes LPA-induced platelet aggregation, and suggested that Sph-1-P and LPA share a common receptor(s) in platelets [10]. Very recently, Gueguen et al. confirmed this and further revealed a precise distinction in platelet-activating activities among various structurally related lysophospholipid molecules [36]. In that work, they also searched expression of Edg family mRNAs in human platelets by RT-PCR, but failed to detect

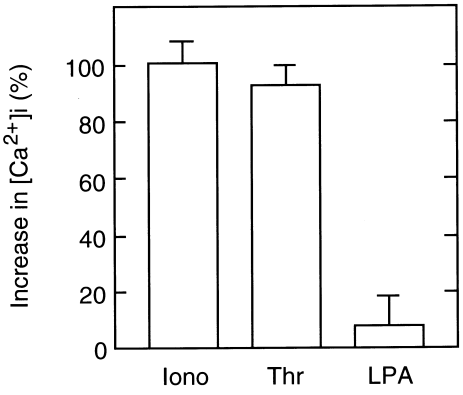

Fig. 3. Desensitization of LPA-induced platelet intracellular Ca^{2+} mobilization by prior addition of Sph-1-P. Platelets supplemented with 1 mM EGTA were preincubated with 40 μM Sph-1-P for 1 min. The cells were then stimulated with 50 nM ionomycin (Iono), 0.04 U/ml of thrombin (Thr), or 5 μM LPA, and the increases in $[Ca^{2+}]_i$ (the ratio of fura2 fluorescence) were measured. The results are expressed as a percentage of control (without Sph-1-P pretreatment). The ratio of fura2 fluorescence of control experiments (100%) was 1.52 ± 0.10 (ionomycin), 1.47 ± 0.06 (thrombin), and 1.47 ± 0.15 (LPA). Columns and error bars represent the mean ± S.D. ($n = 3$).

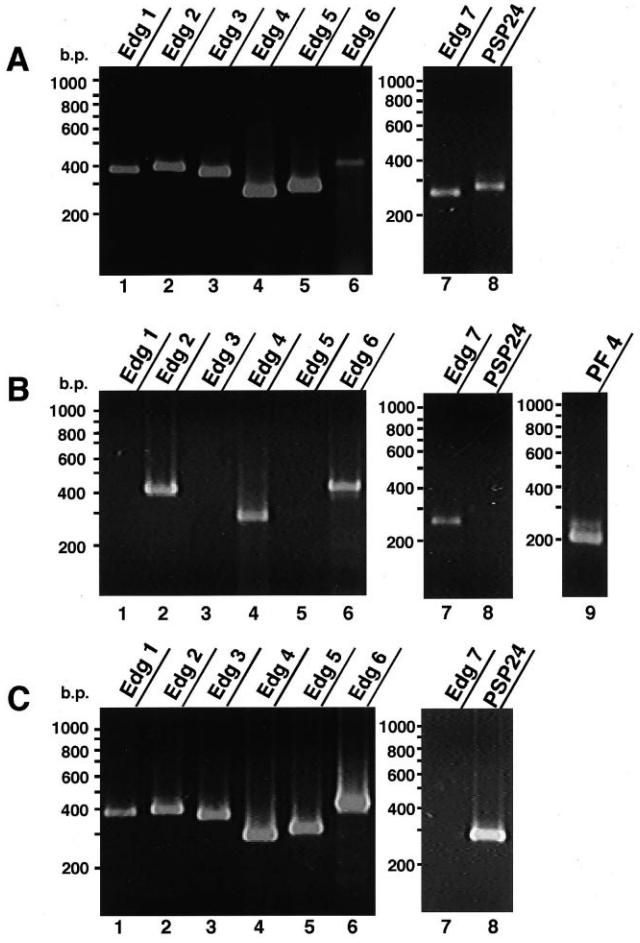

Fig. 4. Expression of Edg family and PSP24 mRNA in various cells. A: RT-PCR for Jurkat mRNA. B: RT-PCR for human platelet mRNA. C: RT-PCR for human PMN leukocytes mRNA. PF4 represents platelet factor 4. Detailed experimental conditions are described in Section 2.

any (Edg1 to Edg4 were examined). Based upon these preliminary experiments, they suggested that lysophospholipid receptors in human platelet might not be a member of the Edg family, but rather an unknown, new type of receptor(s), with a comment that they could not exclude a technical failure in the RT-PCR method.

In this work, we first showed that LPA acts as a strong agonist for platelet aggregation and intracellular Ca^{2+} mobilization, but Sph-1-P as a weak one. Secondly, we demonstrated that Sph-1-P desensitized LPA-induced intracellular Ca^{2+} mobilization, which is consistent with results of desensitization experiments on platelet aggregation [10,36]. These results further suggested that Sph-1-P and LPA may share cell surface receptor(s). We claimed in the previous paper that Sph-1-P itself is a platelet-activating agonist, but the desensitization experiments in this study rather intimate an alternative concept that Sph-1-P released from activated platelets may play a regulatory role toward LPA-induced platelet activation. Thirdly, and most importantly, the expressions of Edg2, Edg4, Edg6 and Edg7 were revealed for the first time in human platelets by RT-PCR with properly prepared mRNA from highly purified platelet preparations. Although at present we cannot completely exclude the possibility that the new type of so far unidentified lysophospholipid receptor(s) can bind both LPA and Sph-1-P, as Gueguen et al. suggested [36], the most likely possibility deduced from our results is that one (or more) of the LPA receptors among Edg2, Edg4 and Edg7 expressed in human platelets may bind Sph-1-P with a low affinity and may function as a common lysophospholipid receptor. The idea is consistent with the results of platelet aggregation and intracellular Ca^{2+} mobilization, in which LPA acts more strongly than Sph-1-P (Figs. 1B and 2). The second possibility is that Edg6, the ligand of which has not been identified yet, may function as a Sph-1-P receptor in a functional collaboration with LPA in human platelets.

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