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理事長　　中島　　章　殿

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Ⅱ．過去の研究歴

Ⅲ．過去の研究実績

1. 楊　麗波、服部　晃ら：黄連解毒湯の血小板凝集・放出能、形態、活性化血小板の発現に及ぼす影響．臨床と研究・69巻9号：314-316, 1992.
2. 楊　麗波、服部　晃ら：黄連解毒湯の血小板の形態、機能、活性化血小板の発現に及ぼす影響．第54回日本血液学会，平成4年4月9-11日・東京.
3. 楊　麗波、布施一郎、服部　晃ら：黄連解毒湯の血小板機能に及ぼす影響－統報－．第34回　日本臨床血液学会，平成4年11月5-7日・大阪.
4. 楊　麗波、布施一郎、服部　晃ら：血小板の adrenalin　potentiation に関する形態学の研究－第一報－．第56回　日本血液学会，平成6年5月11-13日・新潟.
5. Libo Yang, Akira Hattori et al: Studies on Platelet Adrenalin Potentiation. The Third Japanese-Chinese Symposium on Coagulation, Fibrinolysis, and Platelets. Held in Kobe, Japan, from October4-6, 1994.
6. 楊　麗波、服部　晃：散乱光による血小板測定の検討．第一報．第57回　日本血液学会，平成7年6月29-7月1日・名古屋.
7. 楊　麗波、服部　晃：散乱光を利用した血小板凝集計 PA-100の検討　第二報．第18回　日本血栓止血学会、平成7年11月30-12月1日・福岡.

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

1. 楊　麗波、矢冨　裕ら：血小板活性化反応における Sphingosine 1-Phosphate と Lysophosphatidic acid の相互作用．第58回日本血液学会，平成8年4月17-19日・宇都宮.
2. 楊　麗波、矢冨　裕ら：血小板におけるスフィンゴ脂質の代謝．第19回　日本血栓止血学会，平成8年11月6-7日・甲府.
3. 楊　麗波、矢冨　裕、尾崎由基男ら：血小板におけるセロトニンとエピネフリンの相乗作用．第二回関東甲信越セロトニン研究会、平成9年1月18日・東京.

(2) 学会誌等に発表した論文　　無 ・ (有)　（雑誌名・論文名）

別紙参照

Ⅴ．今後の研究計画及び希望

山梨医科大学大学院に入学してより二年間、活性化血小板の検出と血小板活性化機構という、血栓止血学の重要テーマに関して研究を行い、英文誌発表を含め、着実に成果をあげている。大学院卒業までの残り２年間も、同テーマに関し、精力的に研究を続けていきたい。

Ⅵ．研　究　報　告（日本語、又は英語で書いて下さい。 2,000字程度で記載して下さい。）

1．活性化血小板の検出：散乱光を用いた血小板凝集能測定

動脈硬化症や血栓症の発症における、血小板の重要な役割は広く認識されている。その点で、生体内の活性化血小板を客観的に評価することが、きわめて重要と考えられている。従来より使用されている、透過光を用いた血小板凝集測定法は、感度が悪く、血栓形成傾向症例においても、血小板機能の亢進を評価することは困難であった。

当教室では、散乱光を用いた新しい血小板凝集計を開発し、その臨床応用を試みている。この方法を用いると、非常に感度良く血小板凝集塊の生成を検知することができ、活性化血小板の検知、抗血小板剤の評価等にたいへん有効であることが確認されている。

今回、我々は、この鋭敏な方法を用い、作用は弱いものの生理的アゴニストとして重要であるセロトニンとエピネフリンの相乗作用の機序を解析した（研究会発表３）。

さらに現在、各種抗血小板剤の血小板凝集能に対する影響を、散乱光法と透過光法の両者で、詳細に比較検討を行っている。

2．血小板活性化機構

a. 血小板蛋白質チロシンリン酸化反応の解析

血小板における、蛋白質チロシンリン酸化反応の重要性は広く認められているものの、具体的な機能との関連については不明の部分が多く、血小板研究の重要な課題である。当教室では、非受容体型チロシンキナーゼである Syk に関して詳細な検討を続けている。今回、我々は、リゾリン脂質である、スフィンゴシン１リン酸とリゾフォスファチジン酸による Syk の活性化を初めて報告した（別紙発表論文１）。

現在、Syk と Src の無傷血小板における結合様式を、GST 融合蛋白質を用いて検討している。

b. 血小板におけるスフィンゴ脂質の代謝

スフィンゴ脂質は、細胞増殖・分化やアポトーシスにおける重要な作用が報告されているが、血小板における機能・代謝には不明の点が多い。

今回、我々は、スフィンゴシン、セラミド、ジメチルスフィンゴシンの代謝を、アイソトープ標識化合物を用いて検討した。スフィンゴシンは、血小板活性化の有無にかかわらず、速やかにスフィンゴシン１リン酸に代謝される。ジメチルスフィンゴシンは、血小板をトロンビン等で刺激した時のみ、リン酸化される。C_2－セラミドは安定で、代謝を受けなかった（学会発表2）。

以上の研究を行うにあたり、日本財団補助金による 1996 年度日中医学協力事業助成のご援助をいただきました。厚く、御礼申し上げます。

Ⅶ．指導教官の意見

本研究者（楊 麗波）は，現在，山梨医科大学臨床検査医学大学院２年生である。これまで，活性化血小板の検出，血小板活性化機構の解明という，血栓止血学の重要テーマに取り組んできた。研究態度は良好であり，連日，実験・勉学に励んでいる。既に，一流英文誌に５報（うち筆頭者として１報）の論文を発表し，現在も新たに論文投稿準備を進めている。残り２年間において，さらに研究者としての発展が期待できる。

1. Satoh, K., Ozaki, Y., Asazuma, N., Yatomi, Y., Qi, R., Kuroda, K., Yang, L., and Kume, S. Differential mobilization of tyrosine kinases in human platelets stimulated with thrombin or thrombin receptor agonist peptide. *Biochem. Biophys. Res. Commun.* 225: 1084-1089, 1996.

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別紙

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 229, 440–444 (1996)
ARTICLE NO. 1822

Activation of Protein-Tyrosine Kinase Syk in Human Platelets Stimulated with Lysophosphatidic Acid or Sphingosine 1-Phosphate

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It has been reported that not only lysophosphatidic acid (LPA) but also its sphingolipid counterpart, sphingosine 1-phosphate (Sph-1-P), induce platelet functional responses. We report here Syk activation in human platelets stimulated with these lysophospholipids. LPA rapidly induced platelet protein-tyrosine phosphorylation, including that of Syk, and Syk activation, assessed by immunoprecipitation kinase assay. Sph-1-P, although rather weaker, mimicked LPA in inducing these tyrosine kinase-related events. Pretreatment of platelets with staurosporine, a potent protein kinase inhibitor, diminished LPA-induced Syk phosphorylation and activation, but not intracellular Ca^{2+} mobilization. These results demonstrate that, in platelets, the bioactive lysophospholipids induce Syk activation, which, however, may not be related to Ca^{2+} mobilization.

Blood platelets play a central role in hemostasis, while overactive platelets are implicated in the pathogenesis of thrombosis and atherosclerosis. Investigation of the mechanism(s) for platelet activation is important for therapeutic purposes and is expected to have general implications for other cell types, as a model system for working out stimulus-response coupling pathways. Platelets can be activated by a variety of agonists, and lysophosphatidic acid (LPA), the simplest natural glycerophospholipid, is one of the established platelet agonists (1, 2). Furthermore, this bioactive phospholipid is released from activated platelets and is present in serum at physiologically relevant concentrations (3). Therefore, LPA is assumed to act as a local mediator, following discharge from platelets, to regulate cellular functions in an autocrine (or paracrine) fashion (3). Recently, sphingosine 1-phosphate (Sph-1-P), a lysosphingolipid structurally similar to LPA, has also been added to the list of bioactive lipids, capable of activating platelets and released from activated platelets (4, 5). Although the role of these lysophospholipids as autocrine stimulators of platelet aggregation has been proposed (1, 3, 4), the signaling pathway(s) by which they activate platelets are ill-defined.

In this study, we investigated the biochemical mechanisms of platelet activation induced by these lysophospholipids with reference to tyrosine phosphorylation-related events, especially Syk activation, since an increasing body of evidence has suggested an important role of Syk for platelet activation (6, 7).

MATERIALS AND METHODS

Materials. Sph-1-P was prepared from sphingosylphosphocholine with bacterial phospholipase D as described previously (8). The following materials were obtained from the indicated suppliers: LPA and enolase (Sigma, St. Louis, MO); staurosporine (Kyowa Medex, Tokyo, Japan); prostaglandin E_1 (PGE_1) (Funakoshi, Tokyo, Japan);

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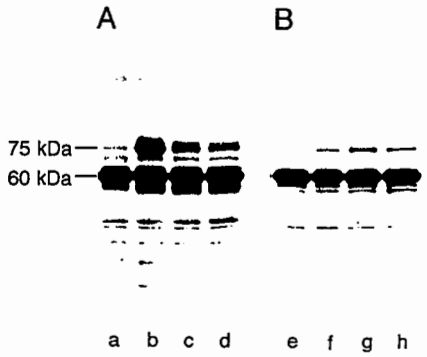

FIG. 1. Platelet protein-tyrosine phosphorylation induced by LPA or Sph-1-P. Human platelets were stimulated with 40 μM LPA (A) or Sph-1-P (B) for 0 s (a and e), 15 s (b and f), 1 min (c and g), and 5 min (d and h). Platelet protein lysates were separated by SDS-PAGE, and tyrosine-phosphorylated proteins were detected by Western blotting using anti-phosphotyrosine MoAb (4G10).

monoclonal antibody (MoAb) against phosphotyrosine (4G10) (Upstate Biotechnology, Lake Placid, NY); MoAb against Syk (Wako Chemicals, Tokyo, Japan); fura2-AM (Dojin Chemicals, Kumamoto, Japan).

Human platelets were isolated from the blood of healthy adult volunteers and handled as described previously (7).

Immunoblot analysis. Platelets proteins were separated, electroblotted, and probed with MoAb against phosphotyrosine or Syk as described previously (7).

Immunoprecipitation kinase assay. Immunoprecipitation of platelet lysates with MoAb against Syk was performed as described previously (7). The immunoprecipitates were used for immunoblotting, as described above, or processed further for *in vitro* kinase assay. *In vitro* kinase assay, using acid-treated enolase as exogenous substrate, was performed as described previously (7).

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ measurement was performed with the use of Ca^{2+}-sensitive fluorophore fura2 as described previously (7), except that 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 (9). The data shown in this study are those obtained form platelets supplemented with 1 mM EGTA to abolish the influx of extracellular Ca^{2+}.

RESULTS

We first examined platelet protein-tyrosine phosphorylation induced by LPA or Sph-1-P (Fig. 1). Among a set of tyrosine-phosphorylated proteins which appeared upon stimulation with LPA, a 75 kDa protein was most heavily and rapidly tyrosine-phosphorylated one (Fig. 1A). Its tyrosine phosphorylation reached maximum as early as 15 s, and then decreased in intensity thereafter. Sph-1-P also induced tyrosine phosphorylation mainly of a 75 kDa protein, but with a slower time course (Fig. 1B). Since the 75 kDa protein band had been suggested to include Syk (and cortactin), based on our previous study using collagen-induced platelets (10), we investigated whether Syk was phosphorylated on tyrosine residues upon stimulation with these phospholipids. Syk was immunoprecipitated with anti-Syk MoAb and analyzed on immunoblots using anti-phosphotyrosine MoAb (Fig. 2). As expected, tyrosine phosphorylation of Syk increased after stimulation of platelets with LPA (Fig. 2A) or Sph-1-P (Fig. 2B). The level of tyrosine phosphorylation paralleled that of a 75 kDa protein observed in Fig. 1.

We next studied Syk tyrosine kinase activity using acid-treated enolase as an exogenous substrate. The enolase phosphorylation activity of Syk increased upon stimulation with LPA (Fig. 3A) or Sph-1-P (Fig. 3B). In these experiments, the amounts of immunoprecipitated Syk were not affected by stimulation of platelets with LPA or Sph-1-P (data not shown). Thus, it is likely that the increase in the enolase phosphorylation activity in the immunoprecipitates was due to an increase in the specific activity of Syk.

From the results described above, platelets were confirmed to undergo Syk phosphorylation

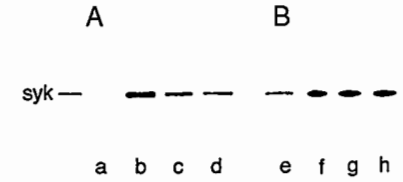

FIG. 2. Tyrosine phosphorylation of Syk in platelets stimulated with LPA or Sph-1-P. Platelets were stimulated with 40 μM LPA (A) or Sph-1-P (B) for 0 s (a and e), 15 s (b and f), 1 min (c and g), and 5 min (d and h). Platelet protein lysates were immunoprecipitated with anti-Syk MoAb. Immunoprecipitates were then subjected to Western blotting using anti-phosphotyrosine MoAb.

and activation upon stimulation with the lysophospholipids (LPA and Sph-1-P). It is well established that intracellular Ca^{2+} mobilization plays a pivotal role in platelet activation induced by most agonists, including LPA (2) and Sph-1-P (4). Since the effect of LPA was stronger than that of Sph-1-P, we evaluated the involvement of Syk activation in intracellular Ca^{2+} mobilization, using LPA-stimulated platelets. For this purpose, we used staurosporine, a very powerful inhibitor of protein kinases (including tyrosine kinases) (11); we previously found that staurosporine was the strongest inhibitor of tyrosine kinase(s) in platelets, although its effect was not so specific (12). LPA-induced whole platelet tyrosine phosphorylation (Fig. 4A), Syk tyrosine phosphorylation (Fig. 4B), and Syk kinase activation (Fig. 4C) were all inhibited by pretreatment of platelets with staurosporine (1 μM). In contrast, intracellular Ca^{2+} mobilization induced by LPA was only slightly inhibited by this treatment (Fig. 5). This protein kinase inhibitor inhibited the Ca^{2+} mobilization by 36.9 $\pm$ 1.2% (mean $\pm$ SD, n = 3), while PGE_1, which inhibits platelet activation by elevating intracellular cAMP (13), completely inhibited LPA-induced intracellular Ca^{2+} mobilization (Fig. 5, lower panel).

DISCUSSION

The lysophospholipids LPA and Sph-1-P, as bioactive lipids, are attracting much interest. Recently, their role as autocrine stimulators of platelets has been proposed, since they are released from activated platelets and their exogenous addition results in platelet activation (1-5). However, the mechanism by which these lipids activate platelets is ill-defined. In this study, we found that Syk, the non-receptor tyrosine kinase which is selectively expressed in hematopoietic cells (6), is tyrosine-phosphorylated and activated by LPA and Sph-1-P. To our knowledge, this is the first to show Syk involvement in the LPA- or Sph-1-P-mediated cell signal transduction. This is probably because elucidation of the cell signaling induced by these lysophospholipids has been performed mainly in non-hematopoietic cells such as fibroblasts and smooth muscle cells (where Syk is not expressed) (1, 14). It is now considered that LPA acts on its cognate G protein-coupled receptor(s), apparently present in many different cell

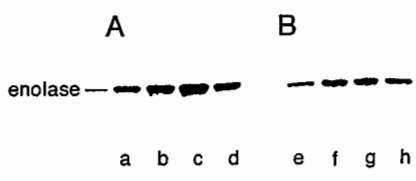

FIG. 3. Activation of Syk in platelets stimulated with LPA or Sph-1-P. Platelets were stimulated with 40 μM LPA (A) or Sph-1-P (B) for 0 s (a and e), 15 s (b and f), 1 min (c and g), and 5 min (d and h). Platelet protein lysates were immunoprecipitated with anti-Syk MoAb. Immunoprecipitates were then subjected to *in vitro* kinase assay using enolase as exogenous substrate.

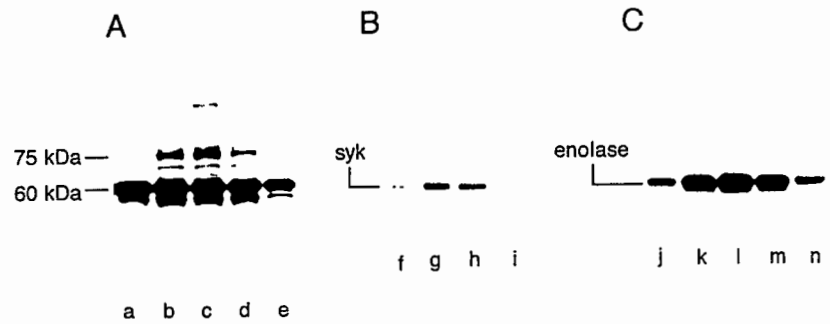

FIG. 4. Inhibition by staurosporine of LPA-induced platelet protein-tyrosine phosphorylation, Syk tyrosine phosphorylation, and Syk activation. Platelets were preincubated for 5 min in the absence (a, b, f, g, j, and k) or presence of 10 nM (c and l), 100 nM (d, h, and m) and 1 μM (e, i, and n) staurosporine and then stimulated without (a, f, and j) or with (b-e, g-i, and k-n) 40 μM LPA. Lysate samples were processed for protein-tyrosine phosphorylation (A), Syk tyrosine phosphorylation (B), and Syk activation (*in vitro* kinase assay using enolase as exogenous substrate) (C). Incubation times of LPA were 15 s in (A) and (B), and 1 min in (C).

types (1). Based on cross-linking experiments, a putative LPA receptor with an apparent molecular mass of 38-40 kDa has been reported in LPA-responsive cells and mammalian brain (15). As for Sph-1-P, the site of action is controversial; some researchers have postulated the existence of cell surface receptor for this phosphorylated Sph (16, 17), while others have proposed Sph-1-P as an intracellular second messenger (18, 19). Elucidation of the mechanism

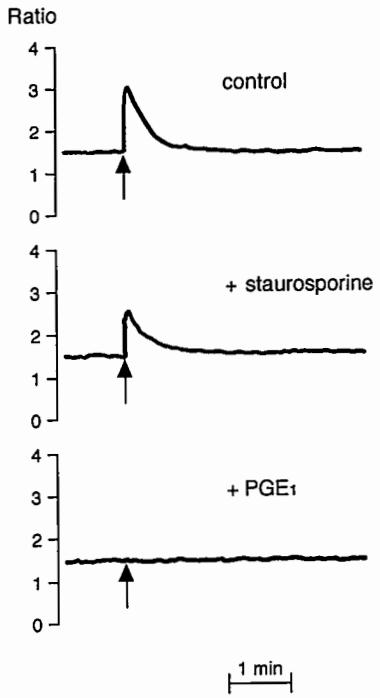

FIG. 5. Effects of staurosporine or PGE_1 on LPA-induced intracellular Ca^{2+} mobilization. Fura2-loaded platelets were preincubated for 5 min in the absence (upper panel) or presence of 1 μM staurosporine (middle panel) or PGE_1 (lower panel), and stimulated with 20 μM LPA. Changes in intracellular Ca^{2+} concentration were measured as described under Materials and Methods. Arrows indicate the addition of LPA.

for Syk activation in signal transduction initiated by cell surface receptors for LPA (and Sph-1-P) may provide an insight into the signaling mediated by these lysophospholipids, which would have general implications for other cell types.

Although the information on Syk-mediated downstream signaling has been fragmentary, Syk involvement in intracellular Ca^{2+} mobilization has been suggested in platelets (6, 20). This has been demonstrated using platelets stimulated with PAF (platelet-activating factor) (20), which is also an important lipid mediator, as well as LPA and Sph-1-P, involved in physiological platelet activation (13). In this context, it should be noted that staurosporine failed to abolish Ca^{2+} mobilization induced by LPA in spite of its inhibition of Syk phosphorylation and activation, suggesting the discrepancy between Syk activation and Ca^{2+} mobilization in LPA-stimulated platelet activation. It seems that various lipid mediators activate platelets in their own ways.

ACKNOWLEDGMENT

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