Identification of lyso phosphatidylthreonine with an aromatic fatty acid surrogate as a potent inducer of mast cell degranulation

Takayuki Kishi\textsuperscript{a,1}, Hiroki Kawana\textsuperscript{a,1}, Misa Sayama\textsuperscript{b,1}, Kumiko Makide\textsuperscript{a,c}, Asuka Inoue\textsuperscript{a,c}, Yuko Otani\textsuperscript{b}, Tomohiko Ohwada\textsuperscript{b,⁎}, Junken Aoki\textsuperscript{a,d,⁎⁎}

\textsuperscript{a} Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan
\textsuperscript{b} Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
\textsuperscript{c} PRESTO, Japan Science and Technology Agency, Japan
\textsuperscript{d} AMED-CREST, Japan Agency for Medical Research and Development, Japan

\textsuperscript{⁎}⁎ Corresponding author at: Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan.

E-mail address: jaoki@m.tohoku.ac.jp (J. Aoki).

\textsuperscript{⁎⁎}⁎ Corresponding author.

\textsuperscript{1} These authors equally contributed to this work.

ARTICLE INFO

Keywords:
Mast cell
Degranulation
Receptor
Lyso phosphatidylserine
Lyso phospholipid

ABSTRACT

Upon various stimulations, mast cells (MCs) release a wide variety of chemical mediators stored in their cytoplasmic granules, which then initiates subsequent allergic reactions. Lyso phosphatidylserine (LysoPS), a kind of lyso phospholipid, potentiates the histamine release from MCs triggered by antigen stimulation. We previously showed through structure-activity studies of LysoPS analogs that LysoPS with a methyl group at the carbon of the serine residue, i.e., lyso phosphatidylthreonine (LysoPT), is extremely potent in stimulating the MC degranulation. In this study, as our continuing study to identify more potent LysoPS analogs, we developed LysoPS analogs with fatty acid surrogates. We found that the substitution of oleic acid to an aromatic fatty acid surrogate (C3-ph-p-O-C11) in 2-deoxy-1-LysoPS resulted in significant increase in the ability to induce MCs degranulation compared with 2-deoxy-1-LysoPS with oleic acid. Conversion of the serine residue into the threonine residue further increased the activity of MC degranulation both in vitro and in vivo. The resulting super agonist, 2-deoxy-LysoPT with C3-ph-p-O-C11, will be a useful tool to elucidate the mechanisms of stimulatory effect of LysoPS on MC degranulation.

1. Introduction

Mast cells (MCs) play a critical role in immediate-type allergic reactions triggered by antigen binding–induced cross-linking of IgE-bound FcεRI (a high-affinity receptor for IgE) and the resulting release of chemical mediators such as histamine and serotonin from their secretory granules, a process known as MC degranulation \cite{1,2}. The released histamine from MCs can cause allergic diseases such as pollinosis, urticaria, atopic dermatitis, and asthma. Identification of factors that modulate MC degranulation would be helpful in providing tools to investigate the molecular mechanisms of allergic reactions as well as to develop anti-allergic drugs.

Lyso phosphatidylserine (LysoPS; 1-acyl-2-lyso-PS or 1-lyso-2-acyl-PS) has several biological activities, including a promotion of neurite outgrowth, a suppression of T lymphocyte proliferation and an enhancement of MC degranulation \cite{3}. Among them, the most characterized biological action of LysoPS is its action to MCs. Exogenous phosphatidylserine (PS) or LysoPS strongly enhances the degranulation of rat peritoneal MCs (RPMCs) initiated by FcεRI cross-linking \cite{4,5}. Because LysoPS is ~1000 times more active than PS and LysoPS is readily produced from PS, LysoPS is considered as the true effector for MCs \cite{5}. The action of LysoPS is highly specific; all other lysophospholipids (including lyso phosphatidyl-D-serine, an optical isomer of lyso phosphatidyl-L-serine) are reported to be ineffective \cite{6}. Thus, a specific receptor for LysoPS should exist on the plasma membrane of RPMCs.

Four G protein-coupled receptors (GPCRs) (LPS1/GPR34, LPS2/P2Y10, LPS3/A630033H20Rik and LPS4/GPR174) that were specifically activated by LysoPS have been proposed as candidate MC LysoPS receptors \cite{8,9}. However, they do not appear to be MC LysoPS receptors because none of them reacted with the super agonist, lyso phosphatidylthreonine (LysoPT; see below) \cite{10}. In addition, MCs isolated from LPS1/GPR34-deficient mice were still activated by LysoPS \cite{11}. Thus, the LysoPS receptor on MC has not been identified yet.

For molecular identification of unknown receptor, specific agonists
with potent activity are definitely useful. We previously synthesized a series of chemically modified LysoPS (so-called LysoPS analogs) and tested their ability to promote antigen-induced RPMC degranulation and to activate cloned LysoPS receptors (i.e., LPS1/GPR34, LPS2/P2Y10 and LPS3/GPR174) [10,12,13]. In these studies, LysoPS was regarded as a modular assembly of serine, phosphate, glycerol and fatty acid and modified each module. Indeed, we developed several LysoPS analogs containing simple modifications in individual modules that were found to be potent inducers of MC degranulation, but were incapable of activating the cloned LysoPS receptors. In the first round of our study, modification was focused on the serine and glycerol modules. As a result, a LysoPS analog with an addition of a methyl group at the carbon of the serine residue, i.e., LysoPT, was identified as a potent LysoPS analog in promoting antigen-elicited MC degranulation [10]. In the second round, the modification was focused on the fatty acid module and a number of LysoPS analogs with fatty acid module and aromatic fatty acid modifications were generated [12,13]. Here, a number of these analogs were evaluated for their ability to promote antigen-induced MC degranulation. We identified an aromatic fatty acid surrogate that greatly enhanced the degranulation-promoting activity of LysoPS. By introducing this surrogate into the structure of 2-deoxy-1-LysoPT, we created a super agonist with ~100-fold higher activity than LysoPS.

2. Methods

2.1. Materials

Oleoyl (18:1)-LysoPS was purchased from Avanti Polar Lipids. LysoPS analogs except 2-deoxy-1-C3-pH-p-O-C11-LysoPT were synthesized as described previously [10,13], and the chemical structure was confirmed by nuclear magnetic resonance (NMR) and element analysis. We confirmed that the purity of LysoPS analogs was always more than 95%. All rats and mice were purchased from Japan SLC.

2.2. Mast cell degranulation assay in vitro

MCs from the peritoneal cavity of rats and mice were prepared essentially as previously described [10]. The MCs were purified by Percoll (GE Healthcare) in HEPES-buffered Tyrode (HBT) solution (pH 7.4) and suspended at a cell density of 5×10⁶/mL in HBT solution containing 0.01% BSA and stimulated with the indicated dose of each LysoPS analog (nM-μM) in the presence of concanavalin A (Sigma Aldrich) (100 μg/mL for RPMC and 10 μg/mL for mouse peritoneal MCs), which is known to cross-link the Fc (Sigma Aldrich) (100 μg/mL for RPMCs and 10 µg/mL for mouse peritoneal MCs), to yield protected, global deprotection with TFA (tri -fluoroacetic acid) and purification by silica gel column chromatography furnished the 2-deoxy-1-LysoPT analog. 2-deoxy-1-C3-pH-p-O-C11-LysoPT as TFA salt (0.67 TFA) satisfied elemental analysis (Purity: > 99.7%). HPLC purity (area normalization method): 95%.

2.3. Evaluation of hypothermic effect of LysoPS analogs

LysoPS and LysoPS analogs were dissolved in PBS containing 0.1% bovine serum albumin and were injected intravenously into mice. Rectal temperatures were measured with an electronic thermometer (Physitemp Instruments) every 5 or 10 min for 70 min.

2.4. TGFα shedding assay

Transforming growth factor α (TGFα) shedding assay was performed as previously described [9]. Briefly, HEK293A (for LPS1, and LPS2) or HEK293FT (for LPS3) cells were transfected with two kinds of plasmids vectors encoding LysoPS receptors and alkaline phosphatase-tagged TGFα. For LPS1, a plasmid vector encoding Gaq/i1 was co-transfected [12,15]. After 24 h, cells were harvested and mixed with a test compound for 1 h. Compound-induced AP-TGFα release from cells was evaluated by determining the AP activity in the conditioned media. Values for AP-TGFα release were presented as the means ± SD for three independent experiments, each in triplicate.

2.5. Synthesis of 2-deoxy-1-C3-pH-p-O-C11-LysoPT

2-deoxy-1-C3-pH-p-O-C11-LysoPT was synthesized from L-threonine, mono-protected propane-1, 3-diol and fatty acid surrogate (C3-pH-p-O-C11) in a similar manner as in [10]: mono-protected propane-1, 3-diol, i.e., 3-((tert-butyldimethylsilyl)oxy)propan-1-ol, synthesized from propane-1,3-diol, was connected with O-phosphanyl N- and C-terminal-diprotected L-threonine, tert-butyl O-(tert-butoxy(diisopro- pylamino)phosphanyl)-N-(tert-butoxycarbonyl)-L-threonine by using phosphoramidite method. After removing the protective group (a tert-butyldimethylsilyl group) from the alcohol moiety, the free hydroxyl group was esterified with the fatty acid surrogate (C3-pH-p-O-C11), to yield protected, Global deprotection with TFA (trifluorocetic acid) and purification by silica gel column chromatography furnished the 2-deoxy-1-LysoPT analog. 2-deoxy-1-C3-pH-p-O-C11-LysoPT as TFA salt (0.67 TFA) satisfied elemental analysis (Purity: > 99.7%). HPLC purity (area normalization method): 95%.

3. Results and discussion

3.1. LysoPS analogs with an aromatic fatty acid surrogate, C3-pH-p-O-C11, is a potent mast cell degranulation inducer

The structure of LysoPS analogs used in this study is shown in (Fig. 1). Because of the ease of chemical synthesis and the fact that they don’t require a sn-2 hydroxy group of LysoPS for MC degranulation-inducing activity, most of the LysoPS analogs tested in this study did not have the sn-2 hydroxy group (deoxy-LysoPS analogs). We replaced the fatty acid of LysoPS with various aromatic fatty acid surrogates (Fig. 1). The LysoPS analogs tested were 2-deoxy-1-C3-pH-o-O-C7-lysoosphatidyldserine (LysoPS), 2-deoxy-1-C3-pH-o-O-C9-LysoPS, 2-deoxy-1-C3-pH-o-O-C11-LysoPS, 2-deoxy-1-C3-pH-m-O-C11-LysoPS and 2-deoxy-1-C3-pH-p-O-C11-LysoPS. Each LysoPS analog (+ a previously characterized deoxy-1-LysoPS (18:1) and LysoPS (18:1)) was tested for their ability to stimulate concanavalin A-induced MC degranulation from RPMCs. The reactivity of each LysoPS analog in stimulating MC degranulation, as judged by histamine release from RPMCs, is shown in Fig. 2. and the results are summarized in Table 1. Modification of the fatty acid module significantly affected the degranulation-stimulating activity of LysoPS. Among the 2-deoxy-1-LysoPS analogs with fatty acid surrogates, 2-deoxy-1-C3-pH-p-O-C11-LysoPS showed the highest activity with an EC₅₀ value about 40 nM (Fig. 2 and Table 1).
3.2. 2-deoxy-1-C3-pH-p-O-C11-LysoPT is the most potent mast cell degranulation inducer

Because conversion of serine polar head to threonine dramatically enhanced the activity to stimulate degranulation reaction of LysoPS [10], we thus synthesized 2-deoxy-1-C3-pH-p-O-C11-LysoPT (Fig. 1) and tested its ability to stimulate degranulation reaction (Fig. 3A and Table 2). The resulting LysoPT analog, 2-deoxy-1-C3-pH-p-O-C11-LysoPT, was extremely potent in stimulating degranulation from RPMCs, with EC₅₀ value about 3 nM. We also confirmed that 2-deoxy-1-C3-pH-p-O-C11-LysoPT was the most potent for inducing degranulation of mouse peritoneal MCs with EC₅₀ value about 3 nM (Fig. 3B and Table 2).

Fig. 1. Chemical structures of LysoPS analogs used in this study.

Fig. 2. Identification of C3-pH-p-O-C11 as the module that confer the greatest MC degranulation activity. LysoPS (18:1) and 2-deoxy-LysoPS analogs with different fatty acid surrogates (Fig. 1) were tested for their ability to induce histamine release from concanavalin A-treated RPMCs. Released histamine, expressed as a percent of the total cell histamine, was determined by fluorometric assay. Values are the means ± SE of four independent experiments, each performed in duplicate.
In this study, to identify a preferable fatty acid module, we used a new set of LysoPS analogs with modification in the fatty acid module [13]. We first identified C3-pH-p-O-C11 as the fatty acid module that conferred the greatest MC degranulation activity (Fig. 2 and Table 1). Introducing the fatty acid surrogate into the structure of a potent ligand, 2-deoxy-1-LysoPT, made it possible to identify a super agonist, i.e., 2-deoxy-1-C3-pH-p-O-C11-LysoPT. Indeed, the activities of the resulting LysoPS analogs showed 100 times higher than LysoPS (18:1) both in vitro (Fig. 3 and Table 2) and in vivo (Fig. 4). The SAR for MC degranulation obtained in this study was different from the SAR for the LysoPS receptors (LPS1, LPS2 and LPS3) [13]. It should be noted that 2-deoxy-1-C3-pH-p-O-C11-LysoPS, a poor inducer of MC degranulation (Fig. 2 and Table 1), was actually a potent agonist for cloned GPCR-type LysoPS receptors [13]. We previously showed that 1-stearoyl (18:0)-LysoPS is a potent agonist for MC degranulation [10], while it was poor agonist for all the cloned GPCR-type LysoPS receptors. By contrast, LysoPS with unsaturated fatty acid such as oleic acid (18:1) and arachidonic acid (20:4) was preferable ligand for cloned GPCR-type LysoPS receptors. Thus, it is reasonable to assume that the ligand recognizing pocket of putative MC LysoPS receptor is quite different from those of cloned GPCR-type LysoPS receptors. Taking account of the fact that the structure of C3-pH-p-O-C11 is more linear than C3-pH-p-O-C11 (Fig. 1), it can be speculated the pocket of the putative MC LysoPS receptor accommodates to linear fatty acids, while those of the cloned GPCR-type LysoPS receptors accommodate to bended or curved fatty acids. We also confirmed that 2-deoxy-1-C3-pH-p-O-C11-LysoPT activated none of the cloned GPCR-type LysoPS receptors in TGFα shedding assay (Fig. 5), suggesting again that the ligand binding manner of MC LysoPS receptor is different from those of the cloned GPCR-type LysoPS receptors.

![Fig. 3](image_url) 2-deoxy-1-C3-pH-p-O-C11-LysoPT is a super agonist for MC degranulation 2-deoxy-1-C3-pH-p-O-C11-LysoPT and related compounds (LysoPS (18:1), LysoPT (18:1) and 2-deoxy-1-C3-pH-p-O-C11-LysoPS for comparison) were tested for their ability to induce histamine release from concanavalin A-treated rat (A) and mouse (B) peritoneal MCs. Released histamine was determined by fluorometric assay and the histamine release is expressed as a percent of the total cell histamine. Values are the means ± SE of three independent experiments, each in duplicate. Each symbol represents; closed circle (•) 2-deoxy-1-C3-pH-p-O-C11-LysoPT, open circle (○) LysoPT (18:1), closed triangle (▲) 2-deoxy-1-C3-pH-p-O-C11-LysoPS and open triangle (△) LysoPS (18:1).

### Table 1

| LysoPS or LysoPS analogs | EC50 | EC50 |
|-------------------------|------|------|
| LysoPS (18:1)           | 150 nM | 80 nM |
| 2-deoxy-1-C3-pH-p-O-C11-LysoPS | > 500 nM | > 500 nM |
| 2-deoxy-1-C3-pH-p-O-C11-LysoPT | > 500 nM | > 500 nM |

### Table 2

| LysoPS or LysoPS analogs | EC50 | EC50 |
|-------------------------|------|------|
| LysoPS (18:1)           | 150 nM | 80 nM |
| LysoPT (18:1)           | 10 nM | 7 nM |
| 2-deoxy-1-C3-pH-p-O-C11-LysoPS | ~ 10 nM | ~ 10 nM |
| 2-deoxy-1-C3-pH-p-O-C11-LysoPT | ~ 5 nM | ~ 5 nM |

3.3. In vivo hypothermic effect

Intravenous injection of 100 µg of LysoPS in mice induced transient hypothermia due to systemic MC degranulation [10]. We focused four LysoPS analogs and LysoPS (18:1) and tested their hypothermic effects. Mice were injected i.v. with the potent LysoPS analogs, and decrease in the rectal temperature was measured. Unlike LysoPS, only 5 or 10 µg i.v. injection of 2-deoxy-1-C3-pH-p-O-C11-LysoPT induced dramatic hypothermia. The rank order matched to that observed for in vitro degranulation-stimulating activity (Fig. 4). Again, 2-deoxy-1-C3-pH-p-O-C11-LysoPT was induced the greatest hypothermic action.

3.4. 2-deoxy-1-C3-pH-p-O-C11-LysoPT did not activate LPS1-3

To examine if 2-deoxy-1-C3-pH-p-O-C11-LysoPT activates LPS1-3, we used TGFα shedding assay. While LysoPS activated all the three LysoPS receptors in TGFα shedding assay, 2-deoxy-1-C3-pH-p-O-C11-LysoPT didn’t (Fig. 5). These results suggest that the putative LysoPS receptor on MCs is different from the cloned GPCR-type LysoPS receptors.

LysoPhosphatidylserine (LysoPS) is composed of four modules, i.e., a serine, a phosphate, a glycerol and a fatty acid, which are chemically linked by phosphodiester or ester bonds. We previously examined structure-activity relationship (SAR) of LysoPS by synthesizing a number of LysoPS analogs with modifications of the four modules, and by evaluating them for both MC degranulation and activation of the cloned GPCR-type LysoPS receptors (LPS1, LPS2 and LPS3) [10,12,13]. Initially our modification was focused exclusively on serine and glycerol modules [10]. As a result, threonine was found to be superior to serine for MC degranulation. In this study, to identify a

![Fig. 3](image_url) 2-deoxy-1-C3-pH-p-O-C11-LysoPT is a super agonist for MC degranulation 2-deoxy-1-C3-pH-p-O-C11-LysoPT and related compounds (LysoPS (18:1), LysoPT (18:1) and 2-deoxy-1-C3-pH-p-O-C11-LysoPS for comparison) were tested for their ability to induce histamine release from concanavalin A-treated rat (A) and mouse (B) peritoneal MCs. Released histamine was determined by fluorometric assay and the histamine release is expressed as a percent of the total cell histamine. Values are the means ± SE of three independent experiments, each in duplicate. Each symbol represents; closed circle (•) 2-deoxy-1-C3-pH-p-O-C11-LysoPT, open circle (○) LysoPT (18:1), closed triangle (▲) 2-deoxy-1-C3-pH-p-O-C11-LysoPS and open triangle (△) LysoPS (18:1).
In summary, we have identified a potent LysoPS analog, 2-deoxy-1-C3-pH-p-O-C11-LysoPT, which induced MC degranulation at nM concentration. The super agonist should be useful not only for pharmacological characterization of the MC LysoPS receptor but also for biochemical identification of the receptor.

Acknowledgements

The present work was supported partly by AMED-CREST (Japan Agency for Medical Research and Development, Core Research for Evolutional Science and Technology) for J.A., PRESTO (Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology) for M.K. and A.I., and Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grant-in-Aid for Scientific Research for J.A. and T.O.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at doi:10.1016/j.bbrep.2016.09.013.

References

[1] C.M. Williams, S.J. Galli, The diverse potential effector and immunoregulatory roles of mast cells in allergic disease, J. Allergy Clin. Immunol. 105 (2000) 847–859.
[2] D.D. Metcalfe, D. Baram, Y.A. Mekori, Mast cells, Physiol. Rev. 77 (1997) 1033–1079.
[3] K. Makide, A. Uwamizu, Y. Shinjo, J. Ishiguro, M. Okutani, A. Inoue, J. Aoki, Novel lysophospholipid receptors: their structure and function, J. Lipid Res. 55 (2014) 1986–1995.
[4] A. Goth, H.R. Adams, M. Knoohuizen, Phosphatidylserine: selective enhancer of histamine release, Science 173 (1971) 1034–1035.
[5] T.W. Martin, D. Laguoff, Interactions of lysophospholipids and mast cells, Nature 279 (1979) 250–252.
[6] H.W. Chang, K. Inoue, A. Bruni, E. Boarato, G. Tofano, Stereoselective effects of lysophosphatidylserine in rodents, Br. J. Pharmacol. 93 (1988) 647–653.
[7] T. Sugo, H. Tachimoto, T. Chikatsu, Y. Murakami, Y. Kikukawa, S. Sato, K. Kikuchi, T. Nagi, M. Harada, K. Ogí, M. Ebisawa, M. Mori, Identification of a lysophosphatidylserine receptor on mast cells, Biochem Biophys. Res Commun. 341 (2006) 1078–1087.
[8] A. Inoue, J. Ishiguro, H. Kitamura, N. Arima, M. Okutani, A. Shuto.
S. Higashiyama, T. Ohwada, H. Arai, K. Makide, J. Aoki, TGFalpha shedding assay: an accurate and versatile method for detecting GPCR activation, Nat. Methods 9 (2012) 1021–1029.

M. Iwashita, K. Makide, T. Nonomura, Y. Misumi, Y. Otani, M. Ishida, R. Taguchi, M. Tsujimoto, J. Aoki, H. Arai, T. Ohwada, Synthesis and evaluation of lysophosphatidylserine analogues as inducers of mast cell degranulation. Potent activities of lysophosphatidylthreonine and its 2-deoxy derivative, J. Med. Chem. 52 (2009) 5837–5863.

I. Liebscher, U. Muller, D. Teupser, E. Engemaier, K.M. Engel, L. Ritscher, D. Thor, K. Sangkuhl, A. Ricken, A. Wurm, D. Pfeiler, S. Schmutzler, H. Fuhrmann, F.W. Albert, A. Reichenbach, J. Thiery, T. Schoneberg, A. Schulz, Altered immune response in mice deficient for the G protein-coupled receptor GPR34, J. Biol. Chem. 286 (2011) 2101–2110.

A. Uwamizu, A. Inoue, K. Suzuki, M. Okudaira, A. Shuto, Y. Shinjo, J. Ishiguro, K. Makide, M. Ikubo, S. Nakamura, S. Jung, M. Sayama, Y. Otani, T. Ohwada, J. Aoki, Lysophosphatidylthreonine analogues differentially activate three LysosPS receptors, J. Biochem. 157 (2015) 2101–2110.

M. Ikubo, A. Inoue, S. Nakamura, S. Jung, M. Sayama, Y. Otani, A. Uwamizu, K. Suzuki, T. Kishi, A. Shuto, J. Ishiguro, M. Okudaira, K. Kano, K. Makide, J. Aoki, T. Ohwada, Structure-activity relationships of lysophosphatidylthreonine analogs as agonists of G-protein-coupled receptors GPR34, P2Y10, and GPR174, J. Med Chem. 58 (2015) 4204–4219.

P.A. Shore, A. Burkhalter, V.H. Cohn Jr., A method for the fluorometric assay of histamine in tissues, J. Pharm. Exp. Ther. 127 (1959) 182–186.

J. Chun, T. Hla, K.R. Lynch, S. Spiegel, W.H. Moolenaar, LXXVIII. Lysophospholipid receptor nomenclature, Pharmacol. Rev. 62 (2010) 579–587.