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PROSPECTS

Metabolic Pathways and Physiological and Pathological Significances of Lysolipid Phosphate Mediators

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Abstract Lysophosphatidic acid and sphingosine 1-phosphate are structurally simple and physiologically very important lysophospholipids. Because they possess distinct structural backbones (glycerol and sphingosine, respectively), there are different metabolic pathways for their intracellular production. Recently, several key enzymes that produce or degrade these lysolipid phosphate mediators extracellularly have been characterized. This review focuses on the physiological and pathophysiological significances of the extracellular metabolic pathways involving recently characterized exo-type lysophospholipase D, ecto-type phospholipase A, and ecto-type lipid phosphate phosphatase. J. Cell. Biochem. 92: 869–881, 2004. © 2004 Wiley-Liss, Inc.

Key words: lysophosphatidic acid; lysophosphatidylcholine; lysophospholipase D; phospholipase A_1; lipid phosphate phosphatase; sphingosine 1-phosphate; sphingosylphosphorylcholine

Lysophosphatidic acid (1 or 2-radyl-*sn*-glycerol-3-phosphate, LPA) has long been believed to be a physiologically important phospholipid [Tokumura, 1995]. The discovery of LPA receptor genes by several groups strongly accelerated not only an advance in LPA research but also the expansion of studies on the orphan G-protein-coupled receptor family for possible phospholipidic ligands. Multiple receptors have been characterized for sphingosine 1-phosphate (S1P), the structure of which resembles that of LPA [Fukushima et al., 2001]. Lysophosphatidylcholine (LPC) and sphingosylphosphorylcholine (SPC) have recently been considered as specific signaling molecules, and several orphan G-protein-coupled receptors have been suggested as their specific receptors, although there is still much debate on this issue [Xu, 2002; Im, 2004].

Consistent with the multiplicity of lysophospholipid receptors, several distinct metabolic pathways seem to exist and to be relevant to the wide distribution of lysophospholipid mediators throughout the animal body. In this review, the physiological and pathophysiological significances of these metabolic pathways and related processes as signaling molecules for the lysophospholipids are surveyed. Emphasis is also placed on recently characterized pathways for extracellular production of the lysolipid phosphate mediators LPA and S1P.

QUANTIFICATIONS OF LPA AND S1P IN BIOLOGICAL SAMPLES

To quantify low levels of physiologically active lysolipid phosphate mediators that coexist with abundant lipids in natural samples, convenient, sensitive, and highly selective methods are required. In earlier investigations, the acyl subclass of LPA (acyl LPA) in animal tissues and body fluids was quantified as fatty acid methyl esters by gas-liquid chromatography after its purification by thin-layer chromatography (TLC). LPA is a unique phospholipid that can be directly analyzed by gas chromatography-mass spectrometry as a volatile trimethylsilyl derivative without any hydrolytic pretreatment [Tokumura, 1995]. Because the fragmentation pattern in the mass spectrum of the alkyl subclass of LPA (alkyl LPA) derivative was distinct from that of acyl LPA, this

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technique was successfully applied to detect small amounts of alkyl LPAs coexisting with more abundant acyl LPAs in rat brain (10.6%) [Sugiura et al., 1999], egg yolk and white (2.4 and 0.2%) [Nakane et al., 2001], and human mixed saliva (11.7%) [Sugiura et al., 2002]. When the selected ion-monitoring mode was used with an appropriate internal standard, low pmol levels of LPAs were quantified by gas chromatography-mass spectrometry [Tokumura, unpublished results]. Further study at the tissue level on alkyl LPA is necessary for assessing its physiological significance, since its as yet uncharacterized specific receptor has been strongly postulated [Tokumura et al., 2002a]. LPA with an arachidonoyl (20:4) group accounts for 5.4% of the total LPA (15.7 nmol/g) in rat brain, and has a positional isomer composition (*sn*-1-20:4-LPA: *sn*-2-20:4-LPA = 37:63) [Nakane et al., 2002]. In structure, the *sn*-2-acyl isomer resembles *sn*-2-arachidonoyl-glycerol (2-AG), an endogenous cannabinoid receptor ligand [Sugiura and Waku, 2000]. In a rat brain homogenate, *sn*-2-20:4-LPA is rapidly converted to 2-AG, indicating its close metabolic relationship to the endocannabinoid in vivo. Similar interconversions are possible between other LPAs and their monoacylglycerols (MG) (Fig. 1), the direction of the reactions depending on the site of the cell and the availability of substrates including ATP. An important problem is whether there is a functional linkage or cross-talk between LPA and endocannabinoid systems in central and peripheral tissues.

Electrospray ionization-mass spectrometry (ESI-MS) combined with TLC [Xiao et al., 2000] or high-performance liquid chromatography (HPLC) [Baker et al., 2001] has also been applied in analysis of LPA from biological samples; the detection ranges by ESI-MS were 5–300 and 2–100 pmol LPA, respectively. By the latter method, 20% of the total LPA in the lipid core of atherosclerotic plaque derived from human carotid artery was found to be the alkyl subclass [Rother et al., 2003]. Using ESI tandem mass spectrometry without purification of LPA by TLC or HPLC (detection limit of 0.06–0.2 pmol), Yoon et al. [2003] found selective increase in the amount of saturated LPAs in plasma from patients with ovarian cancer in comparison with that observed for healthy controls. Another recent approach in analysis of LPA is to quantify the total amount of LPA. In the assay, various LPAs are converted to a common metabolite, phosphatidic acid (PA) with a [^{14}C]oleoyl group (detection limit, 0.2 pmol) [Saulnier-Blache et al., 2000], L-glycerol-3-phosphate analyzed by an enzyme-linked fluorometric assay (detection limit, 0.1 nmol) [Aoki et al., 2002] or a colorimetric assay using an enzymatic cycling method (detection limit, 0.3 pmol) [Kishimoto et al., 2003]. In a sensitive method with a murine homolog of LPA phosphatase, the released inorganic phosphate (detection limit, 2–5 pmol)

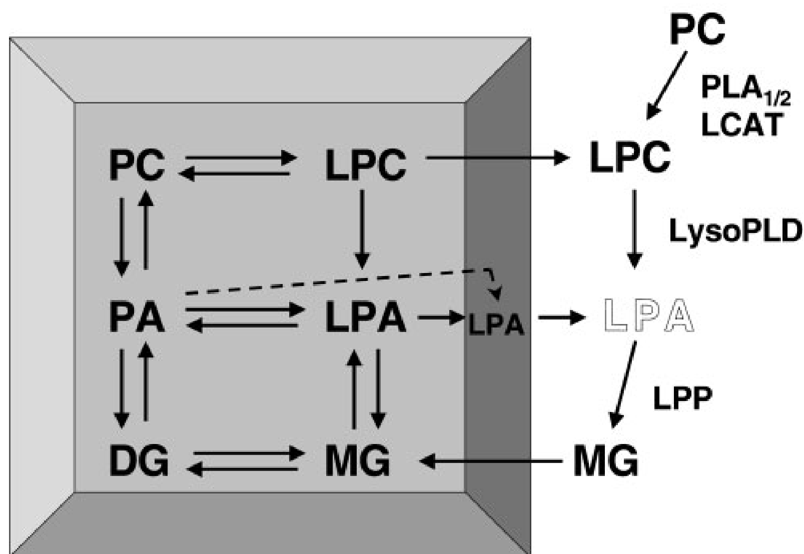

Fig. 1. Intracellular and extracellular metabolic pathways for productions of LPA. DG, diacylglycerol.

was analyzed by coupled enzymatic reactions with maltose phosphorylase, glucose oxidase, and horseradish peroxidase [Luquain et al., 2003]. Although these assays have the advantage of convenience in examination of a large number of biological samples, they do not provide any information on the subclass or molecular species composition.

Because the structure of S1P is simple like that of LPA, there are some difficulties in its quantitative analysis, as reviewed by Okajima [2002]. The first analyses of S1P (100 pmol–10 nmol) were directed to the free hydroxyl group in the sphingosine moiety, which can react with radioactive acetic anhydride [Yatomi et al., 2001]. Dephosphorylation of S1P followed by its re-phosphorylation in the presence of [^{32}P]ATP was used to quantify S1P with the detection limit of 0.25 pmol [Edsall and Spiegel, 1999], and a radioreceptor-binding assay for 2–40 pmol S1P was applied in quantification of S1P in animal sera [Okajima, 2002]. Recent HPLC studies with a derivative of S1P as an *o*-phthalaldehyde form were used to quantify 0.5—100 pmol S1P from biological samples [Ruwisch et al., 2001; Min et al., 2002]. With these methods, the testis, intestine, lung, and spleen were found to be rich-sources of S1P, but its physiological role in these tissues were not unclarified. Certainly, a more selective and sensitive mass spectrometric method is requested for quantification of small amounts of S1P in tissues and body fluids.

CIRCULATING LEVELS OF LYSOPHOSPHOLIPID MEDIATORS

Here, special attention is paid to the circulatory levels of LPA and S1P, as well as recent advances in studies on their sources in the blood circulation. Physiological and pathophysiological levels of two choline lysophospholipids (LPC and SPC) in the blood circulation will be discussed. These choline lysophospholipids may be precursors of the bioactive lysolipid phosphate mediators LPA and S1P, respectively, produced by plasma lysophospholipase D (lysoPLD), although they may exert their own roles as intercellular mediators or sensor molecules in the blood circulation [Xu, 2002; Im, 2004].

LPA

A key enzyme responsible for accumulation of LPA in the blood circulation seems to be lysoPLD that mainly utilizes LPC in the blood [Tokumura, 2002]. Since both serum and heparinized plasma from human subjects or rats contain almost equal activities of lysoPLD, care should be paid not to standing blood collected for preparation of plasma samples for too long. A previous report showed that citrate-based anticoagulants inhibit plasma lysoPLD activity to a small extent, and that EDTA suppresses its activity [Tokumura et al., 1998]. Values for the LPA level in plasma from EDTA-pretreated rat blood (Fig. 2), therefore, can be regarded as not including additional LPA artificially formed after collection of the sample and that they reflect its in vivo circulatory level. As shown in Figure 2, higher values of polyunsaturated LPAs were obtained in citrated and heparinized plasma samples. These results may be explained by the higher production of polyunsaturated LPAs by lysoPLD than that of saturated LPCs during preparation of the plasma.

The level of unknown polar phospholipids with vasopressor activity, probably LPA in the serum of patients with essential hypertension was reported to be higher than that in healthy volunteers [Maebashi and Yoshinaga, 1984]. The same workers found that the production of this vasopressor phospholipid in the serum of rats with spontaneously hypertension (SHR) was more than that in the serum of normotensive Wistar rats. A later study, however, showed that there was no significant difference in the time-dependent production of LPA on incubation of serum from age matched SHR and Wistar–Kyoto rats at 37°C (Table I) [Tokumura et al., 1999a]. Furthermore, the lysoPLD activity of the SHR was compatible to that of Wistar–Kyoto rats (Table I), although values for the two groups were higher than that for conventional Wistar rats [Tokumura et al., 1999a]. Another finding in this study was the age-independence of plasma lysoPLD activity in these strains of rats.

An elevated plasma level of LPA has been postulated to be a marker for early detection of ovarian cancer [Xu et al., 1998]. However, Baker et al. [2002] found no significant difference in the concentrations of LPA in patients with ovarian cancer and healthy subjects. This issue is still a matter of debate, and reasons for the inconsistency are obscure. Because clinical blood samples have often not been very carefully handled, LPA may have been formed by plasma lysoPLD during the handling of blood samples.

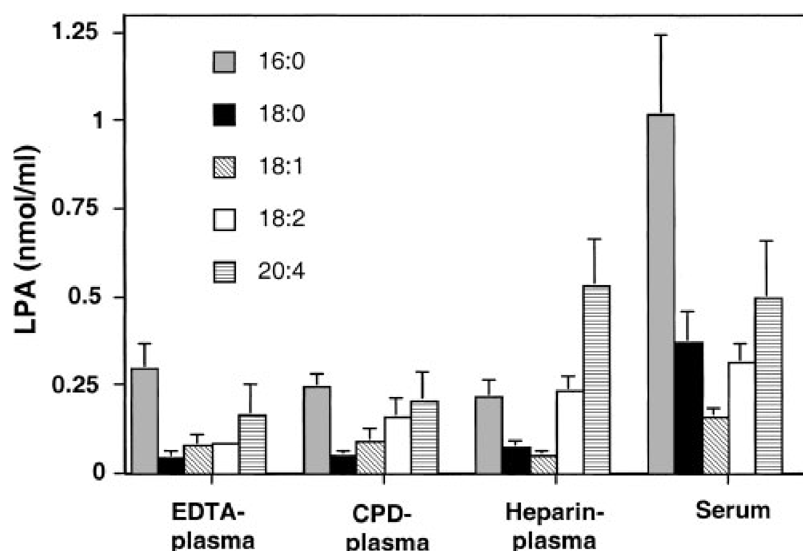

Fig. 2. Levels of major molecular species of LPA in rat serum and plasma derived from EDTA-, CPD-, or heparin-treated blood of rats. Serum was prepared by centrifugation after standing blood samples from male rats (Wistar, 8-weeks-old) at 4°C for 1 day. Samples of rat plasma were prepared at 4°C immediately after mixing of rat blood with 0.1 volume of the citrate/phosphate/dextrose (CPD), EDTA (final, 60 mM), or heparin (final, 25 U/ml) solutions. Lipids were extracted from rat serum and plasma by the method of Bligh & Dyer under acidic conditions, together with 1-heptadecanoyl-LPA, an internal standard, and fractionated by TLC on silica gel plates. LPAs recovered from the silica gel by the method of Bligh & Dyer under acidic conditions, were then quantified by gas chromatography-mass spectrometry as trimethylsilyl ether derivatives, as described previously [Tokumura et al., 2002c]. Values are mean $\pm$ SE of five separate experiments.

In fact, a recent report showing that the activity of serum lysoPLD in patients with ovarian cancer was almost the same as those in healthy subjects and patients with a benign ovarian tumor (Table I) [Tokumura et al., 2002b]. Previous results showing a similar elevated serum level of LPA in patients with multiple myeloma [Sasagawa et al., 1999] may indicate that increase in the LPA level in the blood circulation is not a good selective marker of ovarian cancer.

It is generally accepted that the serum level of LPA is higher than the corresponding level in plasma, consistent with results on the rat shown in Figure 2. Recently, Sano et al. [2002] proposed that extracellular production of LPA in the serum by secretory lysoPLD is greater than its release from activated blood cells and shortly afterwards, Aoki et al. [2002] provided strong evidence for this. Their conclusion was based on firm findings by unique approaches (use of platelet-depleted animals and

TABLE I. Changes in lysoPLD Activity in Mammalian Serum and Plasma in Different Physiological and Pathophysiological Conditions

			Level			
Sample	Condition	LysoPLD activity	16:0-LPC	18:2-LPC	16:0-LPA	18:2-LPA
Human serum	Pregnancy	↗	⇨	↘	↗	⇨
Human serum	Ovarian cancer stages III, IV	⇨	⇨	↘	↗*	↗*
Rabbit serum	High-cholesterol diet (8–12 weeks)	↗	↗	↗	⇨	↗
Rat plasma	Spontaneous hypertension	⇨	⇨	⇨	⇨	⇨

LPA, lysophosphatidic acid; lysoPLD, lysophospholipase D.
Results were reported by Tokumura et al. [1999a, 2002b–d] except for asterisked results [Xu et al., 1998].

patients with deficient blood lecithin-cholesterol acyltransferase (LCAT)- or platelet-activating factor (PAF) acetylhydrolase, and reconstitution of isolated platelets with recombinant lysoPLD). Despite their different approaches, these groups found that various lysophospholipids other than LPC can act as good substrates for serum lysoPLD. So, these pathophysiological environments that resemble that in serum may be attained at local sites in the blood in vivo. Definitely, extensive studies on this topic should be conducted. The potencies of the physiological activities of LPA in the blood circulation would depend on both its local concentration and the density of its receptors on blood and vascular cells, the former being affected by the activities of LPA-producing enzymes, the levels of its precursors, the activities of LPA-degrading enzymes, and the abilities of LPA-binding proteins. Increased generation of LPA in the blood would disturb homeostasis of blood/vascular interaction, leading to more thrombotic and coagulatory conditions. A recent report showed that platelet aggregation induced by oxidized low-density lipoprotein (LDL), a key induction factor of atherosclerosis, was mainly due to the presence of LPA in oxidized LDL. This finding seems suggestive for the potential role of LPA in stimulated formation of thrombi under conditions of increased oxidative stress [Siess, 2002]. Increased in vitro production of LPA in incubated serum was observed with hypercholesterolemic rabbits, but the level of only linoleoyl (18:2)-LPA was greatly elevated despite the great elevation of the serum levels of all molecular species of LPC in this animal model of atherosclerosis (Table I) [Tokumura et al., 2002c]. While no plausible explanation for this unexpected result has yet been provided, it is possible that 18:2-LPC is more resistant than other LPCs to trapping by increased concentrations of lipoproteins as possible reservoirs for circulating LPCs.

Serum lysoPLD activity gradually increases throughout human pregnancy, whereas the level of LPC decreases slightly (Table I) [Tokumura et al., 2002d]. The physiological significance of the elevated lysoPLD activity in the blood circulation during pregnancy is unclear. The involvement in fetal–maternal interaction through the blood–placenta barrier may be necessary for maintenance of pregnancy until term. LPAs produced in the circulation would be supplied continuously to peripheral tissues to exert their diverse physiological effects. The most important enzymes for LPA degradation in the blood circulation are the lipid phosphate phosphatase (LPP) family expressed on the surface of blood cells and vascular cells (Fig. 1) [Brindley et al., 2002]. There is evidence that this family is involved in attenuation of cellular signaling attained by LPA [Brindley et al., 2002; Brauer et al., 2003; Smyth et al., 2003].

In rat plasma, most of the LPA was recovered in the lipoprotein-deficient fraction prepared by ultracentrifugation, and only a small portion of LPA was detected in the high-density lipoprotein (HDL) fraction [Tokumura et al., 1999b]. A recent study reported that the content of LPA in human HDL was about one-fifth of that of S1P [Malchinkhuu et al., 2003]. Putative LPA receptors on human platelets seem unique in their high sensitivity to albumin [Tokumura et al., 2002a], like LPA_3, but not LPA_1 and LPA_2 [Hama et al., 2002]. The albumin-inhibitable aggregation of human platelets in response to LPA is interesting in relation to the pathophysiological role of LPA produced in the blood circulation in vivo. The concentration of the freely diffusible pool of LPA to its putative receptors on the platelets, but not the bulk concentration of LPA, is a determinant of its participation in blood clotting. Thus, it should be determined how much increase in its blood level is required to overcome the masking effect of plasma proteins on LPA-induced effects. In this sense, it should be mentioned that seminal plasma is suggested to limit the physiological availability of the effective pool of LPA to LPA_1 and LPA_2 in the testis, and the identities of the active species remains to be determined [Hama et al., 2002].

LPC

The circulating level of LPC is rather high in various mammals. For instance, it is the second main phospholipid in rat plasma. In most animals, LPCs having a saturated long chain acyl group are more abundant than those having a monounsaturated or polyunsaturated long chain acyl group. The polyunsaturated LPCs are known to be formed in the liver of rats [Brindley, 1993]. Blood albumin is suggested to participate in the hepatic release of LPC, because cultured rat hepatocytes secreted LPC enriched with polyunsaturated fatty acids by a

flop movement of LPC generated within the cells across the plasma membrane [Brindley, 1993]. It is known that saturated LPCs in various animals are formed in blood plasma by the action of LCAT (Fig. 1). Another LPC-producing enzyme in the blood plasma is lipoprotein-associated PAF-acetylhydrolase which selectively hydrolyzes phosphatidylcholine (PC) having an *sn*-2-oxidatively shortened fatty acyl chain [Tokumura, 1995].

The physiological significance of circulating LPC is largely unknown, but preferable incorporation of highly unsaturated LPCs into brain tissues has been demonstrated [Lagarde et al., 2001]. The exact mechanism of LPC passage across the blood–brain barrier is unknown, and there is no firm evidence for the direct uptake of intact LPC. Possibly, soluble lysoPLD is involved in this process together with LPP, providing both MG and choline. The MG would be rapidly internalized probably via its flip movement through the plasma membranes. This hypothesis is worthy of examination. In this context, high expression of mRNA of autotaxin (ATX) in the choroid plexus should be mentioned [Goding et al., 2003].

S1P/SPC

The serum and plasma levels of S1P in humans were estimated to be 0.4–1.2 μM, and 0.2–0.5 μM, respectively [Okajima, 2002]. An elevated serum level of S1P was found in patients undergoing coronary angiography, and so was proposed to be a significant predictor of coronary artery disease [Deutschman et al., 2003]. The circulating level of S1P would be determined by the balance of its release from blood cells and its uptake by vascular and blood cells, and its extracellular degradation by LPPs. Some of the S1P released into the bloodstream might be sequestered in lipoproteins and also be associated firmly with soluble proteins such as albumin, limiting its bio-availability as an intercellular messenger but inhibiting its degradation or uptake by blood and vascular cells. In human plasma, more than 60% of the S1P was shown to bind to lipoproteins, predominantly HDL [Okajima, 2002], in contrast to LPA, which is mainly recovered in the lipoprotein-deficient fraction [Tokumura et al., 1999b]. The physiological significance of S1P in the blood circulation is largely unknown, although HDL-associated S1P was suggested to have prolonged anti-atherogenic effects on the cardiovascular system [Okajima, 2002]. SPC was found in an HDL-associated form in rabbit plasma, but its source is unknown [Liliom et al., 2001].

IDENTIFICATION OF LysoPLD IN THE BLOOD CIRCULATION

Lysophospholipases C and lysoPLD could both be joined lipid phosphodiesterase groups, although their structures and the genes were undetermined until recently. However, identification of lysoPLD in human plasma [Tokumura et al., 2002e] and fetal calf serum [Umezu-Goto et al., 2002] as ATX, a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family that hydrolyzes nucleotides extracellularly [Bollen et al., 2000; Goding et al., 2003], has received much attention from many workers in several research fields. Other members of the E-NPP family (NPP1, NPP3, and NPP5) appear to have no significant lysoPLD activity [Gijsbers et al., 2003; Ohe et al., 2003].

Human plasma lysoPLD, with no HKD motif, is a soluble form of ATX from teratocarcinoma (NPP2β) [Tokumura et al., 2002e]. It has two N-terminal regions, and therefore, a longer subunit having two somatomedin B-like domains and a catalytic domain including Thr^{210} is postulated to be connected to a smaller subunit with a nuclease-like domain possibly through an S-S linkage (Fig. 3), because its treatment with dithiothreitol caused a reduction in the mass of lysoPLD [Tokumura et al., 2002e]. At present, the functional role of the smaller peptide chain is unknown, but it may participate in increase in catalytic function. An example of such a structure for increasing catalytical activity is soluble phospholipase D (PLD) from *Streptomyces chromofuscus*, a metallophosphodiesterase with no HKD motif that can hydrolyze lysophospholipids as well as diacyl-phospholipids [Geng et al., 1999]. Judging from its amino acid sequence which is close to that of the N-terminal region of the longer subunit, furin, a subtilisin-like endoprotease that recognizes Arg-X-Arg (Lys)-Arg-Y- and hydrolyzes the Arg-Y bond, would be involved in hydrolysis of the precursor membrane-associated protein, possibly during transport at the trans-Golgi network. This may be related to the fact that ATX (NPP2) tends to become soluble in contrast to NPP1 and NPP3, which predominantly exist in an ecto-form [Bollen

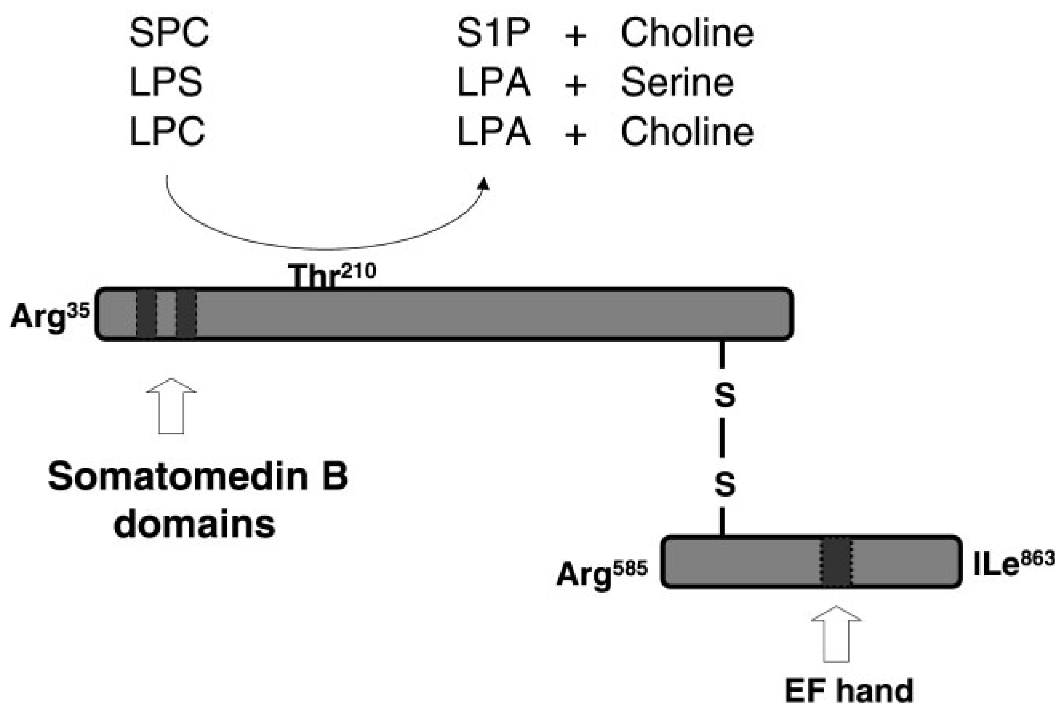

Fig. 3. Schematic representation of hypothetical structure of soluble ATX/lysoPLD isolated from human plasma. LPS, lysophosphatidylserine.

et al., 2000; Gijsbers et al., 2003]. Like *Streptomyces chromofuscus* PLD having Fe^{3+} and other metal ions including Mn^{2+} and Zn^{2+} [Zambonelli and Roberts, 2003], ATX/lysoPLD was suggested to have a dimetal ion site for its activity [Bollen et al., 2000]. Plasma ATX/lysoPLD is sensitive to *o*-phenanthroline, and its activity is potentiated by Co^{2+} [Tokumura et al., 1998, 2002e].

ATX was originally found as a tumor motility-stimulating factor in conditioned medium of human melanoma cells (NPP2α) [Stracke et al., 1992] where lysoPLD activity was later detected [Umezu-Goto et al., 2002]. Another subtype (NPP2γ) was found in glial cells [Fuss et al., 1997]. ATX/lysoPLD could play a critical role in the pathophysiology of cancer [Mills and Moolenaar, 2003]. Beside its known tumor motility-stimulating effect that has now been recognized to be due to its lysoPLD activity to produce LPA with potent bioactivity on various carcinoma cells [Umezu-Goto et al., 2002], ATX was found to stimulate the motility response of normal cells such as human coronary artery smooth muscle cells in vitro and stimulate new blood vessel formation in vivo [Bollen et al., 2000]. These findings seem important in relation to the well-known effects of S1P on angiogenesis and a recent finding that ATX/lysoPLD is able to convert SPC to S1P with 4.5-fold lower catalytic efficacy than that for egg LPC [Clair et al., 2003]. In addition, ATX/lysoPLD was found to hydrolyze various lysophospholipids other than LPC [Aoki et al., 2002]. Thus, its bioactions may be expressed dependent on the type of available substrate and its local environmental situation. Little is known about the extracellular level of SPC.

The source of circulating ATX/lysoPLD in the blood stream is unknown, although the mRNA of ATX was detected in human umbilical venous endothelial cells, coronary artery smooth muscle cells, aortic smooth muscle cells, human bronchial epithelial cells, fibroblast-like synoviocytes and rat choroid plexus epithelial cells, and the secretion of ATX from human umbilical venous endothelial cells was reported to be stimulated by basic fibroblast growth factor [Bollen et al., 2000]. An early study showed that abnormally low serum levels of a vasopressor phospholipid (probably LPA generated by plasma lysoPLD) in both laterally nephrectomized dogs and patients with chronic renal failure, suggesting that the kidney may be the source of lysoPLD in the blood circulation [Maebashi and Yoshinaga, 1984]. Ferry et al. [2003] reported that ATX/lysoPLD

released from differentiated adipocytes hydrolyzed LPC secreted from both adipocytes and preadipocytes to produce LPA, which is capable of accelerating the proliferation of preadipocytes in adipose tissues in vivo. Thus they suggest that adipocytes are a source of plasma ATX/lysoPLD.

PRODUCTION OF LYSOPHOSPHOLIPID PHOSPHATE MEDIATORS IN BIOLOGICAL FLUIDS

LPA was detected in ascitic fluid from ovarian cancer patients [Xiao et al., 2001; Baker et al., 2002], cerebrospinal fluid of an experimental model of hemorrhage injury, rabbit aqueous humor, and lacrimal gland fluid of the eye [Tigyi and Parrill, 2003], follicular fluid from patients programmed in vitro fertilization [Tokumura, 2002], adipose tissue microdialysates [Valet et al., 1998], hen egg white and yolk [Nakane et al., 2001], and human mixed saliva [Sugiura et al., 2002]. At present, the sources of LPA in these biological fluids are unknown. Possibly, it is secreted from cells, but more likely it is generated extracellularly from secreted lysophospholipids by lysoPLD [Tokumura, 2002], as in the case in the blood described above. Baker et al. [2002] reported that ascitic fluid from patients with ovarian cancer had higher levels of LPA (9.77 ± 4.79 μM) than control subjects with nonmalignant diseases (1.83 ± 1.45 μM). They found that the fatty acid composition of plasma-derived LPA (18:2-rich) differed from that of ascitic fluid-derived LPA (16:0-rich), suggesting a lack of exchange between two fluids. The relative lysoPLD activity on the 16:0-LPC relative to that on 18:2-LPC was higher for the ascitic-derived lysoPLD than for the serum-derived lysoPLD (Tokumura A, unpublished data). LPA is known to induce invasion of several types of carcimoma cell, and therefore, biological fluids having unusually high enzymatic activity of LPA production (for example, ascites from patients with malignant tumors) would give microenvironments with a high potential of metastasis of cancers [Umezu-Goto et al., 2002; Mills and Moolenaar, 2003].

Several body fluids including hen egg white were found to have various extents of lysoPLD activity, and the properties of lysoPLD in certain body fluids were not identical to those of plasma ATX/lysoPLD in terms of substrate specificity, susceptibility of augmentation of activity by Co^{2+}, and sensitivity to *o*-phenanthroline. In particular, lysoPLD in human mixed saliva and amniotic fluid is insensitive to *o*-phenanthroline (Tokumura A, unpublished data). Further studies are needed to characterize the structures of novel secreted lysoPLDs in various biological fluids. It will be interesting to examine what types of phospholipids are physiological and pathological substrates for the phenanthroline-sensitive and -insensitive lysoPLDs. Their elevated levels, not the activity of lysoPLD, could limit the rate of LPA production at local sites under pathological states. Third, the uncharacterized lysoPLDs mainly exist in exo-forms, not ecto-forms, like plasma ATX/lysoPLD. Why is this? Does the bathing of a wide surface area of tissue by body fluid with more LPA have any benefits on the tissues? There are also many other questions to be answered.

The alkenyl subclass of LPA (alkenyl LPA) was reported to be present in rabbit aqueous humor fluid and lacrimal gland fluid of the eye and in human ascitic fluid [Xiao et al., 2001; Tigyi and Parrill, 2003; Xu et al., 2003]. Alkyl LPA was detected in human mixed saliva at 0.104 μM (11.7% of the total LPA) [Sugiura et al., 2002] and in ascitic fluid from patients with ovarian cancer at about 0.1 μM [Xiao et al., 2001]. The source and target for alkyl-LPA or alkenyl LPA in the body fluids are of interest.

Ascites from patients with ovarian cancer contains S1P in a concentration range of 0.42–2.17 μM, but another group detected 51.4 nM S1P in ascitic fluid from patients with ovarian cancer [Xu et al., 2003]. Human amniotic fluid was found to contain 19 nM S1P, and its level to be increased about 2-fold during labor [Kim et al., 2003]. Interestingly, these workers showed that S1P modulated cyclo-oxygenase-2 expression in human amnion-derived WISH cells. However, little is known about the source of S1P in biological fluids, although SPC, the precursor of S1P for lysoPLD, was also shown to be present in ascitic fluid from patients with ovarian cancer at 38.9 nM [Xu et al., 2003]. The possibility of extracellular generation S1P from SPC in animal fluids should be examined.

RELEASE OF LYSOLIPID PHOSPHATE MEDIATOR FROM ANIMAL CELLS

Lysolipid phosphate mediators are amphiphilic and thus would spread from their generation site to surrounding tissues on their release into extracellular fluids containing lipid-binding proteins. Furthermore, some of

the lysophospholipids generated in intracellular membranes would be transported to the plasma membrane, and finally become exposed to the extracellular space by transmembrane movement, endocytic membrane fusion and other as yet unknown mechanisms.

Increase in the extracellular level of LPA might be mediated by its increased production or decreased decomposition in intracellular and extracellular compartments, as well as by its accelerated release from the cells. Activated platelets were shown to release LPA by an unknown mechanism, and secretory phospholipase A_2 (PLA_2)-IIA was suggested to be involved in the production of LPA in vesicle microaggregates derived from platelets [Gaits et al., 1997]. In the case of adipocytes, its extracellular production appears to be a major mechanism for its increase in conditioned medium, since the differentiated adipocytes were demonstrated to release both lysoPLD and its substrate, LPC [Ferry et al., 2003]. On the other hand, the constitutive release of LPA formed within the cells has been suggested to account for its increase in extracellular level during culture of ovarian carcinoma cells [Mills and Moolenaar, 2003]. Phorbol myristate acetate (PMA), Ca^{2+} ionophore, LPA, and nucleotides stimulated the rapid release of LPA in PLD- and phospholipase A_1/A_2 (PLA_1/A_2)-dependent fashions from ovarian cancer cell line SK-OV-3 (Fig. 1), although this cell line secreted relatively high lysoPLD activity [Mills and Moolenaar, 2003]. Regardless of its actual metabolic pathway, unusually increased activity of LPA-producing enzyme would allow some local extracellular spaces to be pathophysiologically sensitive to LPA. For instance, ascitic fluid from patients with ovarian cancer is of high metastatic potential for ovarian carcinoma cells [Mills and Moolenaar, 2003]. Very recently, laminin, an extarcellular component of the matrix, was found to increase the level of LPA (up to 110 nM) in the supernatant of cultures of HEY ovarian cancer cells [Sengupta et al., 2003]. Interestingly, the increase in the levels of 18:1- and 18:2-LPA were much higher than those of other acyl-LPAs as well as alkyl-LPAs, although these authors postulated Ca^{2+}-independent PLA_2 as a key enzyme for LPA production.

The most interesting enzyme responsible for formation of LPA as an intercellular mediator seems to be membrane-bound PLA_1 selective for PA (mPA-$PLA_1\alpha$ and mPA-$PLA_1\beta$) predominantly expressed in human platelets [Sonoda et al., 2002] and sperm [Hiramatsu et al., 2003], respectively (Fig. 1). These authors stressed that LPA production by this enzyme pathway may contribute to the local accumulation of LPA in microenvironments such as those after tissue injury, because of the rapidity of LPA generation. Another important finding in their study was the formation of LPA from PA in the outer layer of the plasma membrane from which LPA might approach a binding site of its specific receptor. An unsolved problem is how PA generated by intracellular PLD becomes accessible to membrane-associated PA-specific PLA_1 the catalytic site of which locates in the outer layer of the plasma membranes.

The decreased ecto-expression of LPP-1 or LPP-3 is relevant to the elevated accumulation of extracellular LPA and attenuation of LPA-induced activating effects on ovarian cancer cells in vitro and in vivo [Mills and Moolenaar, 2003]. Similarly, differentiation of preadipocytes into mature adipocytes was demonstrated to be associated with decrease in the hydrolytic activity of the cells on extracellular LPA [Simon et al., 2002]. A postulated new member of the LPP family expressed in neuronal cells, plasticity-related gene-1, was recently reported to be involved in axon growth in developing brain of fetal mice and regenerative sprouting in adult mice, possibly degrading surrounding LPA and affecting neurite retraction. In this connection, the mechanism of the attainment of postulated LPA-rich local environments in the central nervous system should be clarified. Depending on the local situation in the body, either lysoPLD activity or LPP activity or both may be altered, affecting the extracellular level of the lysolipid phosphate mediators.

S1P

Since intracellular metabolic pathways for S1P and related release mechanisms have been documented in an excellent review [Yatomi et al., 2001], only recent advance on this topic is addressed here. The level of S1P in platelets was estimated as 1.4 nmol/10^9 cells, corresponding to about 0.5 μM in the blood if all of the stored SIP were released. Constitutive release of S1P from human platelets was found to be rapid, whereas slow release of LPA was observed only after the activation of platelets [Sano et al., 2002]. Both thrombin and PMA stimulated the release of S1P from human platelets in a protein

kinase-dependent manner. Extracellular albumin at 1% was found to accelerate the PMA- or thrombin-induced release of S1P in an uncharacterized fashion [Yatomi et al., 2001]. A measurable amount of SIP was slowly released from immunoglobulin E plus antigen-stimulated mouse mast cells [Prieschl et al., 1999], whereas 0.4 nM of S1P in the conditioned medium of human umbilical vein endothelial cells was suggested to be due to its extracellular production by exported sphingosine, but not its release from the cells [Ancellin et al., 2002]. It will be interesting to examine whether a similar mechanism operates to provide a biologically effective pool of S1P.

TRANSMEMBRANE MOVEMENTS OF LYSOPHOSPHOLIPID MEDIATORS

Flop Movement

The cystic fibrosis transmembrane regulator is a member of the ATP binding cassette family, and was recently postulated to function as a flippase for S1P and LPA [Boujaoude et al., 2001]. Their interesting study was designed on the basis of the high percentage homology between this mammalian ABC transporter and a member of the ABC family encoded by a yeast oligomycin resistance gene that appears to pump S1P out of yeast, thus acquiring resistance to sphingosine. A major finding was the significantly higher uptakes of radioactive S1P and LPA by murine C127 epithelial cells stably transfected with the wild-type human cDNA of cyctic fibrosis transmembrane regulator than those by mock-transfected control and ΔF508 mutant C127 cells. However, in this study there was no report of structural analysis of the cell-associated radioactive lipid. In other words, there was no conclusive evidence that the radioactive probe taken up into the cells without its metabolic conversion. Both S1P and LPA are known to be hydrolyzed by LPPs in a wide range of cells [Brindley et al., 2002], so the above finding might be interpreted by supposing that over-expression of the cystic fibrosis transmembrane regulator indirectly causes increased hydrolyses of S1P and LPA on the cell surface to the more cell-permeable metabolites, sphingosine and MG.

Flip Movement

Intracellularly produced LPA may exert its intracellular actions by binding to specific intracellular proteins such as the peroxisome proliferator-activated receptor-γ [McIntyre et al., 2003]. A recent report documenting the existence of a nuclear LPA_1 receptor that is involved in the regulation of pro-inflammatory genes is also interesting [Gobeil et al., 2003]. Since the experimental results suggesting the intracellular action of LPA were obtained with intact cells exposed to exogenous LPA, one may wonder how LPA can reach the intracellular target sites from outside the cell. However, physiologically, the intracellular production of LPA should be more closely related to its intracellular action, than its extracellular production. An early attempt to elucidate the mode of internalization of LPA across the plasma membrane of intact animal cells was performed using the albumin back-extraction procedure to monitor radioactive alkyl LPA and its acetylated analog [Tokumura et al., 1992]. Alky LPA and its analog were quickly adsorbed to the outer layer of the plasma membrane of guinea pig neutrophils, but the rate of its apparent internalization, monitored by the albumin back extraction procedure, was slower than that of alkyl MG. Parallel analysis of radioactive lipid revealed that much of the internalized radioactivity was recovered in metabolites such as triacylglycerol, phosphatidylethanolamine and PC together with minor portions in alkyl LPA and alkyl MG. These results suggest that exogenously added alkyl LPA was degraded, probably by LPP activity, on the cell surface, and the resultant alkyl MG rapidly diffused into the inner layer of the plasma membrane. Although internalized MG was promptly metabolized by several enzymes, a small, but significant portion of the rephosphorylated alkyl LPA from alkyl MG was accumulated within the cells. While details of these processes are unknown, these results indicated that the intracellular level of alkyl LPA remained high for more than 30 min after its exogenous addition to intact neutrophils.

Future Scope

The physiological roles of LPA and S1P in mammals have yet to be determined. In particular, studies are required on their distribution, synthesis, metabolism, and transmembrane movement. The mechanisms of the bidirectional movement across biological membranes should also be investigated. Changes in the levels of LPA and S1P and of LPC and SPC in various

biological fluids in different pathophysiological situations should also be studied more. Results should provide an understanding of the pathogeneses of several chronic diseases in which unusual metabolism is involved, and thus in developing therapeutic agents for the diseases.

Recent studies on exo- and ecto-enzymes metabolizing lysolipid phosphate mediators in animal cells revealed that the metabolic pathway for LPA and possibly for S1P in the animal body is more complicated than previously thought. At least, a combination of lysoPLD or PLA_1 and LPP is basically important in the metabolic pathway of extracellular LPA. The enzyme systems possibly upstream or downstream of the key enzymes should be examined. Studies are also needed on whether a similar extracellular metabolic pathway is related to effective generation S1P from SPC by lysoPLD-like activity at local sites in the animal body. The finding and characterization of additional members of the lysoPLD, LPP, and PLA_1/PLA_2, families as well as novel lysophospholipid-binding proteins, are eagarly wanted. Development of inhibitors of the key enzymes is an attractive future research project.

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