Serum Lysophosphatidic Acid Is Produced through Diverse Phospholipase Pathways*

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Lysophosphatidic acid (LPA) is a lipid mediator with multiple biological activities that accounts for many biological properties of serum. LPA is thought to be produced during serum formation based on the fact that the LPA level is much higher in serum than in plasma. In this study, to better understand the pathways of LPA synthesis in serum, we evaluated the roles of platelets, plasma, and phospholipases by measuring LPA using a novel enzyme-linked fluorometric assay. First, examination of platelet-depleted rats showed that half of the LPA in serum is produced via a platelet-dependent pathway. However, the amount of LPA released from isolated platelets after they are activated by thrombin or calcium ionophore accounted for only a small part of serum LPA. Most of the platelet-derived LPA was produced in a two-step process: lysophospholipids such as lysophosphatidyicholine (LPC), lysophosphatidylethanolamine, and lysophosphatidylserine, were released from activated rat platelets by the actions of two phospholipases, group II A secretory phospholipase A2 (sPLA2-IIA) and phosphatidylserine-specific phospholipase A2 (PS-PLA2), which were abundantly expressed in the cells. Then these lysophospholipids were converted to LPA by the action of plasma lysophospholipase D (lysoPLD). Second, accumulation of LPA in incubated plasma was strongly accelerated by the addition of recombinant lysophospholipase D with a concomitant decrease in LPA accumulation, indicating that the enzyme produces LPA by hydrolyzing LPC produced during the incubation. In addition, incubation of plasma isolated from human subjects who were deficient in lecithin-cholesterol acyltransferase (LCAT) did not result in increases of either LPC or LPA. The present study demonstrates multiple pathways for LPA production in serum and the involvement of several phospholipases, including PS-PLA2, sPLA2-IIA, LCAT, and lysoPLD.

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‡ The abbreviations used are: LPA, lysophosphatidic acid; PA, phosphatic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LPS, lysophosphatidylserine; LPE, lysophosphatidylethanolamine; LPs, lysophospholipids; PLA2, phospholipase A2; PLA3, phospholipase A3; EDG, endothelial cell differentiated gene; lysosphopholipase D; PS-PLA2, phosphatidylserine-specific phospholipase A2; sPLA2-IIA, secretory PLA2 group II A; LCAT, lecithin-cholesterol acyltransferase; mPA-PLA2, membrane-bound PA-selective PLA2; PAF-AH, platelet-activating factor acetylhydrolase; MGL, monoglyceride lipase; G3PO, glycerol-3-phosphate oxidase; GPCP, phosphocholine phosphodiesterase; AC2, acid citrate dextrase; FLI, familial LCAT deficiency; C3PLA2, calcium-dependent cytosolic PLAs; IPLA2, calcium-independent cytosolic PLA2; LPI, lysophosphatidylinositol.

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These observations suggest that LPA species are biologically significant and are produced by diverse synthetic pathways. Serum LPA can be produced from phospholipid precursors either in membranes of blood cells or in plasma by sequential actions of phospholipases present in plasma or expressed by blood cells. In rats, two PLAs, secretory phospholipase A2 group II A (sPLA2-IIA) (19, 20) and phosphatidylserine-specific phospholipase A1 (PS-PLA1) (21, 22), are expressed predominantly in platelets (23). We previously showed that these two PLAs are involved in agonist-induced production of lysophospholipids such as LPC, lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS) in activated platelets by analyzing the phospholipid composition using specific inhibitors of sPLA2-IIA (24). In addition, other phospholipases capable of producing lysophospholipids, possibly LPC, have been identified in plasma. Two such phospholipases are lecithin-cholesterol acyltransferase (LCAT) and platelet-activating factor acetylhydrolase (PAF-AH). It has been suggested that part of the LPC present in blood is attributed to the transesterification of phosphatidylcholine (PC) and free cholesterol catalyzed by LCAT (25). It is also possible that the plasma PAF-AH (26) contributes to LPC production by hydrolyzing oxidized phosphatidylcholine (27), which has been implicated in various pathological conditions. We have recently identified the above-mentioned lysoPLD by purifying the enzyme (28). The identification and cloning of these phospholipases make it possible to examine their contribution to serum LPA production. In this study, to elucidate the one or more synthetic pathways for LPA in serum, we attempted to clarify the roles of blood cells, plasma, and the phospholipases in serum LPA production.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-rat platelet serum was purchased from InterCell Technologies Inc. Bovine serum albumin (fatty acid free, A-6003) was purchased from Sigma. 1-Oleoyl (18:1)-LPA, 1-oleoyl-LPC, 1-oleoyl-LPE, 1-oleoyl-LPS, porcine liver LPI, sphingosylphosphocholine, and dioleoyl-PC were purchased from Avanti Polar Lipids Inc. (Alabaster, AL), and 1-[3H]oleoyl-LPA (18:1) was from Amersham Biosciences (Uppsala, Sweden). Monoglyceride lipase (MGL), glycerol-3-phosphate

![FIG. 1.](image1) ![FIG. 2.](image2)
Quantification of LPA and LPC—Concentrations of LPA and LPC were determined by an enzyme-linked fluorometric method established in the present study. LPA concentration was determined by fluorometry of H2O2 using 3-(4-hydroxyphenyl)propionic acid (7.5 nm, Dijin, Tokyo, Japan) as a peroxidase donor (29) generated by the reaction of LPA samples with 10 units/ml monoglycerceride lipase (MGL, Asahi Chemical Industry Co. Ltd., Shizuka, Japan) and 10 units/ml glycerol-3-phosphate dehydrogenase (G3PO, Asahi Chemical Industry Co. Ltd.) was applied in the assay system. The concentrations of LPA were determined from a standard curve, after subtracting the tentative values obtained from the fluorescence intensities of the MGL (+)G3PO (+) reaction from those of the MGL (−)G3PO (−) reaction. Similarly, the concentration of LPC was determined from a standard curve, after subtracting the tentative values obtained from the MGL (+)G3PO (+)G3PO (−) reaction from those of the MGL (−)G3PO (−)G3PO (−) reaction.

LysoPLD Assay—LysoPLD activity was assayed as described previously (28). Briefly, samples (1–50 μl) were incubated with 1 μM LPC (from egg) in the presence of 100 μM Tris-HCl (pH 9.0), 500 μM NaCl, 5 μM MgCl2, and 0.05% Triton X-100 for 1 h at 37°C. The liberated choline was detected by an enzymatic photometric method using choline oxidase (Asahi Chemical, Tokyo, Japan), horse radish peroxidase (Toyo, Osaka, Japan), and TOOS reagent (N-ethyl-N-2-hydroxy-3-sulfopropyl)-3-methylaniline, Dojin, Tokyo, Japan) as a peroxidase donor (29) generated by the reaction of LPA to the samples. Based on recovery of 1-[3H]oleoyl-LPA, lipid recovery was always >95% under the above-described conditions. LPC concentration was determined by a similar method except that 10 units/ml phospholipase (phosphodiesterase (G3PO, Asahi Chemical Industry) was expressed by a baculovirus system as described previously (28, 31). The recombinant proteins were partially purified from the culture supernatant of SF9 cells infected with each baculovirus using heparin and Mono Q column chromatography (Amersham Pharmacia Biotech, Buckinghamshire, UK) and were dialyzed in phosphate-buffered saline (−/−) before use. sPLA2-IA was purified from cell supernatant of thrombin-activated rat platelets as described previously (28, 57).

RESULTS

Role of Platelets in Serum LPA Production—To evaluate the involvement of platelets in the production of serum LPA, we prepared platelet-depleted animals and determined their serum LPA level by a fluorometric method established in this study (see “Experimental Procedures”). The number of platelets in rats treated with rabbit anti-platelet serum was about 1% of that in control animals treated with control rabbit serum (Fig. 1B). The treatment with rabbit anti-platelet serum did not affect the numbers of other blood cells such as erythrocytes and white blood cells (data not shown). Under these conditions, serum LPA levels were 0.94 ± 0.14 μM in control antibody-treated animals and 1.95 ± 0.14 μM in control antibody-treated animals (Fig. 1A). Plasma LPA levels in these animals were 0.17 ± 0.02 μM (platelet-depleted) and 0.16 ± 0.02 μM (control). These results confirmed a previous report that the LPA level is high in serum but low in plasma (8). In addition, the present result shows that half of LPA in serum is produced by a platelet-dependent pathway.

We next examined the contribution of platelets themselves to...
the production of LPA in serum by measuring the LPA produced by isolated platelets upon their activation. As shown in Fig. 1C, rat platelets produced and released LPA after they were activated by thrombin or by a calcium ionophore, A23187. However, the levels of LPA produced by these activators (0.10 ± 0.02 and 0.16 ± 0.01 μM, respectively) were too low to account for the LPA produced by the platelet-dependent pathway of LPA production in serum. The human platelets were also found to have a low ability to produce LPA upon activation (Fig. 2B).

**Lysophospholipids Secreted from Activated Platelets Are Converted to LPA by LysoPLD**—In view of the findings that much of the lysophospholipids, but not LPA, is produced in activated platelets (24) and that lysoPLD activity is detected in plasma of several mammalian species, including rat and human (28, 32), we hypothesized that part of the LPA in serum is produced in two steps: generation of lysophospholipids in activated platelets and their subsequent conversion to LPA by lysoPLD. To test this possibility, we examined whether the full amount of LPA produced in the platelet-dependent pathway is detected when isolated platelets are activated in the presence of lysoPLD. As was observed previously (24), a high concentration of LPC was detected in the supernatant of activated platelets stimulated with thrombin or A23187 (Fig. 2C). The addition of a physiological concentration of recombinant lysoPLD dramatically increased the amount of LPA in the supernatant of the activated rat or human platelets and slightly decreased the LPC level (Fig. 2B). In the presence of recombinant lysoPLD, the concentration of LPA rose to 0.63 ± 0.09 μM and 0.73 ± 0.12 μM when isolated rat platelets were activated by thrombin and A23187, respectively (Fig. 2B). These concentrations are comparable to the concentration produced in the platelet-dependent pathway (Fig. 1). Among various lysophospholipids detected in the activated rat platelets, only LPC has been shown to be a substrate of lysoPLD. However, as shown in Fig. 2A, lysoPLD hydrolyzed other lysophospholipids, such as

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**Fig. 4. LPA production in other blood cells.** Isolated rat erythrocytes (RBC) and white blood cells (WBC) were incubated in the presence or absence of recombinant lysoPLD. LPA and LPC concentrations of the culture cell supernatant were determined. The amount of lysoPLD added was the same as that detected in rat plasma (102 pmol/min.μl). LPA secreted from thrombin-activated rat platelets both in the presence or absence of recombinant lysoPLD is shown for comparison. These data represent the means ± S.D. of three independent experiments.
PLA, LPS, and LPI, to produce LPA. These results clearly indicate that, in the platelet-dependent pathway for LPA synthesis, lysophospholipids produced by activated platelets are converted to LPA by the action of lysoPLD present in blood plasma.

Production of Lysophospholipids by sPLA$_{IIA}$ and PS-PLA$_1$ and Their Subsequent Conversion to LPA by LysoPLD—We further examined the roles of sPLA$_{IIA}$ and PS-PLA$_1$ in lysoPLD-enhanced LPA production in activated rat platelets, because the two PLAs are predominantly expressed in the cells and have been implicated in lysophospholipid production in activated platelets (24). As shown in Fig. 3, addition of recombinant sPLA$_{IIA}$ or PS-PLA$_1$ dramatically increased the LPA production evoked by addition of recombinant lysoPLD from activated platelets stimulated with either thrombin or A23187. This suggests that the two PLAs are involved in serum LPA production through their roles in supplying lysophospholipids to lysoPLD.

**Contribution of Other Blood Cells to Serum LPA Production—**We further determined whether erythrocytes or white blood cells are involved in serum LPA production. To examine LPA production in blood cells, we incubated erythrocytes, white blood cells, and platelets prepared from rat blood with a physiological concentration of recombinant lysoPLD. Of the different cell types examined, platelets were by far the most potent. Of the blood cells, and platelets prepared from rat blood with a physiological concentration of recombinant lysoPLD, LPA production in blood cells, we incubated erythrocytes, white blood cells are involved in serum LPA production. To examine LPA production in blood cells, we incubated erythrocytes, white blood cells, and platelets prepared from rat blood with a physiological concentration of recombinant lysoPLD. Of the different cell types examined, platelets were by far the most potent.

**Involvement of LysoPLD and LCAT in the Production of LPA in Plasma—**Both LPA and LPC are produced in plasma during prolonged incubation at 37 °C in rat (32). As shown in Fig. 5, we confirmed that incubation of plasma or serum both from rat and human at 37 °C increased the accumulation of both LPA and LPC in a time course-dependent manner. The formation of LPA in serum is much faster than that in plasma, especially in the initial period (1–6 h). LPC concentration in serum was slightly higher than that in plasma (Fig. 5), indicating that lysophospholipids accumulated in incubated serum can be derived both from plasma phospholipids and, as a result of blood coagulation, from platelet phospholipids. It is likely that lysoPLD converts these lysophospholipids to LPA. Consistent with this idea, the addition of recombinant lysoPLD to the plasma dramatically increased the formation of LPA and resulted in a smaller increase in LPC (Fig. 6). A similar result was obtained in incubated serum (data not shown). Addition of a divalent cation chelator such as EDTA or EGTA to the incubation medium almost completely inhibited the accumulation of LPA but did not affect the accumulation of LPC (Fig. 6, EGTA data not shown). This observation strongly indicates that LPC present or generated during the incubation in plasma is converted to LPA by lysoPLD activity, and it is compatible with the fact that the enzyme requires a divalent cation for its activity (33).

We further examined the mechanism of LPA formation in incubated plasma. It has been suggested that part of the LPA present in blood is due to the activities of lecithin:cholesterol acyltransferase (LCAT) (25) or platelet-activating factor acetylhydrolase (PAF-AH) activities (27). The formation of LPA during incubation was insensitive to EDTA (see Fig. 8), which is compatible with the properties of the two plasma enzymes (34, 35). To evaluate the contribution of these enzymes to the accumulation of LPC in plasma during incubation, we examined the plasma LPC level of human subjects deficient in LCAT (FLD) or PAF-AH. As was observed in plasma from normal subjects (Fig. 5), the LPC level increased during incubation at 37 °C in the PAF-AH-deficient plasma. On the other hand, LPC did not form at all in the plasma from LCAT-deficient patients (Fig. 7), which confirmed that LCAT is responsible for the accumulation of LPC in the incubated plasma. In addition, it was revealed that LPC accumulation was significantly suppressed in LCAT-deficient plasma but not in the control or PAF-AH-deficient plasma (Fig. 7). The LCAT-deficient plasma had a comparable level of LPC (~100 μM) (Fig. 7), indicating that a part of the plasma LPC can also be produced by an LCAT-independent pathway.

**DISCUSSION**

The present study was undertaken to clarify how LPA is produced in serum. There are at least four synthetic pathways for serum LPA (Fig. 8). The major two pathways identified in the present study are 1) secretion of lysophospholipids such as LPC, LPE, and LPS from platelets, followed by conversion of the lysophospholipids to LPA (Fig. 2) and 2) generation of LPC from PC in lipoprotein and its consequent conversion to LPA (Figs. 6 and 7). The first pathway requires activation of platelets. Thus, LPA is produced through this pathway under pathological conditions, in which platelets are activated. Such pathological conditions are found at sites of injury, inflammation, and atherosclerosis. In contrast, the second pathway is continuously active in blood, which makes it difficult to evaluate it as discussed below. The other two pathways are 3) production by isolated platelets upon their activation (Fig. 1) and 4) production by erythrocytes (Fig. 4). However, these two pathways make minor contributions to serum LPA production.
LysoPLD Is a Key Enzyme in Serum LPA Production

LysoPLD is involved in the first, second, and fourth pathways and thus contributes considerably to LPA production in serum (Fig. 8). Because lysoPLD is the only enzyme that exhibits lysoPLD activity in blood plasma or serum (28), it can be concluded that lysoPLD is a key enzyme in serum LPA production. Recently we have shown that lysoPLD is identical to autotaxin (28). Autotaxin/lysoPLD is an autocrine motility factor that stimulates motility and proliferation of cancer cells (28, 36). The product of lysoPLD, LPA, is an effective inducer of chemotaxis (37–40) and cell proliferation (41) in multiple cell lineages. In addition, serum has multiple cell effects on cell proliferation and motility. Thus, it is possible that some of these biological activities of serum are explained by its lysoPLD activity.

LysoPLD does not appear to be activated as a result of blood coagulation, because its activity in serum and plasma were about the same (data not shown). Thus, lysoPLD seems to be continuously active in blood, which suggests that LPA is continuously produced in the bloodstream through the second and fourth pathways, although the LPA level in fresh plasma is quite low (~0.1 μM). This low level can be explained by our preliminary finding that LPA does not accumulate in plasma when the plasma is incubated in the presence of blood cells.2 There is some evidence that lipid phosphate phosphatases are involved in cellular degradation of lysophospholipid mediators, including sphingosine 1-phosphate and LPA (42, 43). Lipid phosphate phosphatases expressed by blood cells may be responsible for keeping the LPA level lower, resulting in negative regulation of LPA signaling.

LCAT Supplies LPC to LysoPLD in Plasma

The formation of LPC and LPA was decreased in plasma from LCAT-deficient subjects (Fig. 7). Because plasma lysoPLD activities of LCAT-deficient and control subjects were not significantly different, LCAT appears to have a critical role in the second pathway by supplying LPC to lysoPLD (Figs. 7 and 8). LCAT deficiency in humans, referred to as familial LCAT deficiency (FLD), is characterized by a complete lack of plasma LCAT activity, corneal opacification, anemia, proteinuria, and kidney dysfunction (44–46). The plasma of these patients has decreased concentrations of total cholesterol, esterified cholesterol, and high density lipoprotein cholesterol, and increased concentrations of triglyceride, phospholipid, free cholesterol, and very low den-
sity lipoprotein cholesterol. Although most of the phenotypes of LDL patients seem to be explained by abnormal composition and shape of plasma high density lipoprotein particles, some can be explained by the reduced LPA production. We observed that LPC is still present in LCAT-deficient plasma, indicating that LPC originates from several metabolic pathways. It is likely that LPC is generated intracellularly and is also secreted directly by hepatocytes, which are a quantitatively important source of plasma unsaturated LPC (47). Although the molecular mechanisms underlying the LCAT-independent LPC production are still unclear, they may have a role in lysoplasmid-mediated production of LPA, especially LPA with unsaturated fatty acids.

**Lysophospholipids Generation by Diverse Phospholipase As—**Lysophospholipids that are converted to LPA by lysoplasmid can be generated by actions of various phospholipase As. The present study demonstrated that sPLA2-IIA and PS-PLA1, which, in rats, are predominantly expressed in platelets, are involved in the production of serum LPA. We showed that, in rat platelets, these enzymes generate lysophospholipids (24) (Figs. 3 and 8), which are then converted to LPA by the lysoplasmid in plasma. Balle et al. (48) showed that serum LPA production in mice is not affected by either inhibitors of sPLA2-IIA or a phospholipase C. As previously known to have such a role. We conclude that LPA is not be overlooked, because it can generate LPA rapidly and may play a role in certain microenvironments such as sites of injury or hemostasis.

Finally, during the preparation of this report, Sano et al. (54) reported that LPA and sphingosine 1-phosphate are produced in the course of blood coagulation in humans. Based on an analysis of the acyl composition of the LPAAs generated and the fate of exogenously added fluorescent phospholipid analogs, they suggested some possible pathways and enzymatic activities that essentially agree with our results, even though their approach was quite different from ours. Using plasma from genetically deficient patients and recombinant enzymes, we further clarified the involvement of individual lysophospholipids in the production of LPA. These lysophospholipids were not previously known to have such a role. We conclude that LPA is produced in multiple pathways by sequential reactions of phospholipases. Our next challenge is to evaluate the biological significance of each synthetic pathway.

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