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A fluorometric assay for lysosomal phospholipase A2 activity using fluorescence-labeled truncated oxidized phospholipid

Akira Abe[a,*], Miki Hiraoka[a], James A. Shayman[b], Hiroshi Ohguro[a]

[a] Department of Ophthalmology, School of Medicine, Sapporo Medical University, Sapporo, Japan
[b] Department of Internal Medicine, University of Michigan Medical School, University of Michigan, Ann Arbor, MI, USA

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ABSTRACT

Lysosomal phospholipase A2 (LPLA2) is a key enzyme involved in the homeostasis of cellular phospholipids. Recently, LPLA2 was reported to preferentially degrade some truncated oxidized phospholipids at the *sn*-1 position. A commercially available, truncated oxidized phospholipid conjugated with a fluorescent dye, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(dipyrrometheneboron difluoride) butanoyl] (PGPE-BODIPY), was used to develop a specific assay for this enzyme. When recombinant mouse LPLA2 was incubated with liposomes consisting of 1,2-*O*-octadecyl-*sn*-glycero-3-phosphocholine/PGPE-BODIPY under acidic conditions, PGPE-BODIPY was converted to palmitic acid and a polar BODIPY-product. After phase partitioning by chloroform/methanol, the polar BODIPY-product was recovered in the aqueous phase and identified as 1-lyso-PGPE-BODIPY. The formation of 1-lyso-PGPE-BODIPY was quantitatively determined by fluorescent measurements. The *Km* and *Vmax* values of the recombinant LPLA2 for PGPE-BODIPY were 5.64 μM and 20.7 μmol/min/mg protein, respectively. Detectable activity against PGPE-BODIPY was present in LPLA2 deficient mouse sera, but the deacylase activity was completely suppressed by treatment with 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). AEBSF had no effect on LPLA2 activity. The LPLA2 activity of mouse serum pre-treated with AEBSF was specifically and quantitatively determined by this assay method. The PGPE-BODIPY and AEBSF based LPLA2 assay is convenient and can be used to measure LPLA2 activity in a variety of biological specimens.

Introduction

Lysosomal phospholipase A2 (LPLA2) is ubiquitously expressed in mammalian cells and tissues. In particular, phagocytic cells such as macrophages highly express LPLA2 and where the lipase plays a role in the degradation and clearance of intracellular and extracellular materials containing glycerophospholipids [1,2]. Alveolar macrophages from LPLA2 deficient mice develop phospholipidosis [2]. In addition, older LPLA2 deficient mice develop an autoimmune phenotype [3]. LPLA2 is a secreted protein as well as a lysosomal protein [4] and is present in plasma [5] and aqueous humor [6]. Thus, LPLA2-related disorder may be diagnosed by measuring the LPLA2 activity in extracellular fluids.

Previously, we reported a detection method for LPLA2 activity by use of a synthetic fluorogenic phospholipid, a phosphatidylglycerols in which one acyl group is conjugated with one FAM (fluorescein amidite) group and the other to DABCYL [4-(4-dimethylaminophenylazo)-benzoyl] group (1-FAM-2-DABCYL-PG) [7]. The reaction was carried out using liposomes incorporated with 1-FAM-2-DABCYL-PG plus LPLA2 under acidic conditions. One of the reaction products, 1-FAM-2-lyso-PG, was recovered and isolated in the aqueous phase from 1-FAM-2-DABCYL-PG and the remaining reaction products by phase partition using chloroform and methanol. LPLA2 activity was determined by fluorescence intensity of the aqueous phase. The method was used to assess the effect of cationic amphiphilic drugs on LPLA2 activity [7]. However, 1-FAM-2-DABCYL-PG was not an efficient substrate to detect LPLA2 activity in a crude enzyme source such as plasma and serum because the specificity of 1-FAM-2-DABCYL-PG against the enzyme was dependent on the phospholipid composition of liposomes containing 1-FAM-2-DABCYL-PG. Importantly, the solution containing 1-FAM-2-lyso-PG required neutralization to detect the fluorescence of 1-FAM-2-lyso-PG, necessitating an extra step in the assay [7].

Recently, we reported that LPLA2 has a preference for truncated oxidized glycerophospholipids and preferentially cleaves a long chain acyl group at the *sn*-1 position of these phospholipids [8]. Several truncated oxidized phospholipids labeled with a fluorophore are commercially available. These observations led us develop a more convenient and specific fluorogenic assay method for the measurement of

* Corresponding author. Department of Ophthalmology, School of Medicine, Sapporo Medical University, S1W16 Chuo-ku, Sapporo, Hokkaido, 060-8543, Japan.
E-mail address: abeakira@sapmed.ac.jp (A. Abe).

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LPLA2 activity. A truncated oxidized phosphatidylethanolamine (PE) derivative conjugated with a BODIPY fluorophore at the amino group, PGPE-BODIPY, was chosen due to the broad preference of LPLA2 to a polar group of glycerophospholipids. Based on the acyl chain specificity of LPLA2 against the truncated oxidized phospholipids, LPLA2 should convert PGPE-BODIPY to palmitic acid and 1-lyso-PGPE-BODIPY. 1-lyso-PGPE-BODIPY is a more polar lipid than either PGPE-BODIPY or palmitic acid. Therefore, the lyso- PGPE-BODIPY and PGPE-BODIPY/palmitic acid would be recovered into the aqueous phase and the organic phase, respectively, after phase partitioning by chloroform and methanol. In addition, the emission of BODIPY fluorophore is independent of pH. Therefore, the LPLA2 activity assay could be attained by a simple measurement of fluorescence intensity of the aqueous phase as previously required.

In this study, recombinant mouse LPLA2 and mouse serum were used as a source of LPLA2 enzyme. We first investigated whether the fluorogenic truncated oxidized phospholipid properly acts as a substrate for LPLA2 using recombinant LPLA2. We then established a specific fluorescence measurement method of LPLA2 activity using the serum prepared from LPLA2 deficient mice.

Materials and methods

Materials

1-Palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(dipyrromethemeboron difluoride) butanoyl] (PGPE-BODIPY), 1,2-*O*-octadecyl-sn-glycero-3-phosphocholine (DODPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) and *N*-acetylsphingosine (NAS) were obtained from Avanti Polar Lipids Corp. (Alabaster, AL); recombinant mouse LPLA2 was from Proteos (Kalamazoo, MI); HPTLC silica gel plates, 10 × 20 cm, were from Merck (Darmstadt, Germany); 4-(2-aminoethyl)benzenesulfonyl fluoride was from Sigma-Aldrich (St. Louis, MO).

LPLA2 activity on PGPE-BODIPY

For the preparation of PGPE-BODIPY/DODPC liposomes, those lipids (molar ratio of PGPE-BODIPY to DODPC: 1:2.4) were mixed in a glass tube and dried down under a stream of nitrogen gas. DODPC is a non-hydrolyzable phosphatidylcholine. The dried lipid mixture was dispersed into 50 mM sodium citrate (pH 4.5) using a probe-type sonicator for 8 min in an ice water bath.

The reaction mixture consisted of 49 mM sodium citrate (pH 4.5), 10 μg/ml BSA, 38 μM PGPE-BODIPY incorporated into 92 μM DODPC liposomes, and LPLA2 or mouse serum in 500 μl of total volume. The reaction was initiated by adding recombinant LPLA2 or mouse serum, kept for a specific time period at 37 °C and terminated by adding 3 ml of chloroform/methanol (2:1, v/v) plus 0.3 ml of 0.9% (w/v) NaCl (the final ratio of chloroform/methanol/aqueous solution: 2:1: 0.8, v/v). In other cases, one hundred μl of the reaction mixture was taken at a specific time point and mixed with 3 ml of chloroform/methanol (2:1, v/v) plus 0.4 ml of 50 mM sodium citrate (pH 4.5) and 0.3 ml 0.9% NaCl. The mixture containing organic solvents was centrifuged at 800*g* for 5 min at 20 °C. The resultant aqueous phase (upper layer) was collected into a small brown glass vial with a screw cap and kept at room temperature until the fluorescence was measured. The lower organic layer was transferred into another glass tube and dried down under a stream of nitrogen gas.

TLC assay

The dried lipid from the organic phase was dissolved in chloroform/methanol (2:1, v/v), applied to an HPTLC plate and developed in a solvent system consisting of either chloroform/methanol/water (60:35:8, v/v) or chloroform/methanol/pyridine (98:2:0.5, v/v). To visualize the BODIPY fluorophore, the plate was dried and then exposed under ultraviolet (UV) light. In order to detect non-fluorescent products formed in the reaction, the plate was soaked in 8% (w/v) $CuSO_4$, $5H_2O$, 6.8% (v/v) H_3PO_4, 32% (v/v) methanol. The uniformly wet plate was briefly dried using a hair dryer and charred for 15 min in a 150 °C oven. The charred plate was scanned and the content of the product was estimated by NIH-ImageJ 1.37v. For the aqueous phase, each sample was directly applied on an HPTLC plate and then treated as described above.

Fluorescence measurement

The aqueous phase containing the BODIPY product was kept in a brown tube. The BODIPY emission spectrum of the aqueous phase was recorded in the scanning range 500–600 nm using a fluorometer (Hitachi F-2300, Japan). The excitation wavelength was constant at 490 nm. Emission and excitation slits were 5 nm. The fluorescence intensity at the emission peak (506.5 nm) was defined as (the fluorescence intensity obtained from the study in the presence of LPLA2 or serum) - (the fluorescence intensity obtained from the study in the absence of LPLA2 or serum) at each indicated time period in the reaction.

The BODIPY-derivative formed in the reaction is not available as a standard. To measure quantitatively the BODIPY-product, a standard curve was created using a known amount of PGPE-BODIPY, which was dispersed in the aqueous phase solution obtained by the phase partition used in this study. PGPE-BODIPY was freely dispersed in the aqueous phase solution and emitted BODIPY monomer fluorecence as the BODIPY-product. In the present study, we assumed that the quantum yield of PGPE-BODIPY in the aqueous phase solution is not different from that of the BODIPY-product in the same solution.

Results

Degradation of fluorogenic truncated oxidized phospholipid by LPLA2

The molecular structure of PGPE-BODIPY, the fluorogenic truncated oxidized phospholipid tested, is shown in Fig. 1A. DODPC liposomes containing PGPE-BODIPY were incubated with recombinant mouse LPLA2 to confirm whether LPLA2 is able to degrade the fluorogenic phospholipid.

The reaction solution containing PGPE-BODIPY/DODPC liposomes (molar ratio of 1–2.4) prior to the addition of the enzyme showed red orange color and was not fluorescence emitting. However, upon incubation with the LPLA2 a change in the color of the reaction mixture was observed. The color turned from red orange to light green (Fig. 1B-1, right tube). No color change was observed in the reaction mixture in the absence of LPLA2 (Fig. 1B-1, left tube). When the reaction mixtures were exposed under UV light, the reaction mixture containing LPLA2 emitted fluorescence (Figs. 1B-2, right tube). In addition, the fluorescent product formed in the presence of LPLA2 was recovered in the aqueous phase when the reaction mixture was partitioned into two layers using chloroform/methanol/aqueous solution (2:1:0.8, v/v) under acidic conditions (Figs. 1B-3, right tube). By contrast, PGPE-BODIPY was recovered in the organic phase (Figs. 1B-3, left tube).

Thin layer chromatography (TLC) was used to identify the reaction products (Fig. 2). A reaction product was recovered in the organic phase that increased with time and was identified as palmitic acid when compared with an authentic palmitic acid (Fig. 2B and C). Concurrently, PGPE-BODIPY was gradually decreased in the presence of LPLA2 (Fig. 2A). Also, the fluorogenic BODIPY reaction product recovered in the aqueous phase displayed a slower mobility than PGPE-BODIPY and increased with time (Fig. 2D). In addition, the BODIPY-product was hydrolyzed by treatment with diluted alkaline solution (data not shown). One of the resultant products produced by alkaline treatment was a fluorescent compound with mobility between the BODIPY-product and PGPE-BODIPY on TLC. This product had the same mobility as one of the products produced from PGPE-BODIPY by the same alkaline treatment (data not shown). Because weak alkaline

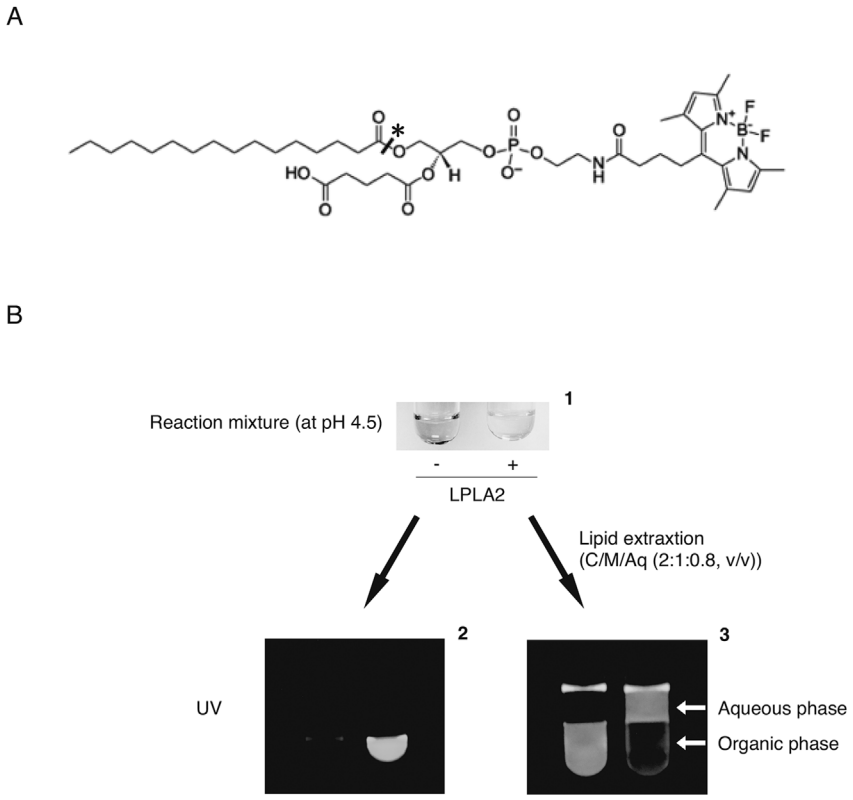

Fig. 1. Reaction of PGPE-BODIPY with LPLA2. Figure **A** shows a chemical structure of PGPE-BODIPY. * denotes a postulated cleavage site of PGPE-BODIPY by LPLA2 (**A**). Liposomes consisting of PGPE-BODIPY/DODPC (molar ratio, 1:2.4, 130 μM as phospholipid concentration) were incubated with or without 4 μg/ml of recombinant mouse LPLA2 for 1 h at 37 °C (**B1**). The reaction mixtures after the reaction were exposed under UV-ray (**B2**) and were mixed with chloroform and methanol (the final ratio of chloroform/methanol/aqueous solution, 2:1:0.8 (v/v)). After the phase partitioning, two layers were completely separated by centrifugation (**B3**).

treatment results in a cleavage of ester linkage in glycerophospholipids, the fluorogenic product formed after the alkaline treatment is glycero-3-PE-BODIPY. These observations strongly support the conclusion that the BODIPY-product found in the aqueous phase is 1-lyso-PGPE-BODIPY.

Dilution of the BODIPY-product in the aqueous phase after the partition by chloroform/methanol/aqueous solution made the quantitative analysis of the BODIPY-product by TLC difficult. However, the diluted product was stable at room temperature in darkness and was measurable by using a fluorometer. When DODPC/PGPE-BODIPY liposomes were incubated with recombinant mouse LPLA2, the fluorescence intensity of the aqueous phase at 506.5 nm by excitation light at 490 nm increased with time (Fig. 2D, E and 2F). These data indicate that the formation of the polar BODIPY-product by LPLA2 increased with time under acidic conditions. These results demonstrate that LPLA2 activity can be measured conveniently and quantitatively using PGPE-BODIPY.

Fluorescence measurement of LPLA2 activity using PGPE-BODIPY

The polar BODIPY-product recovered in the aqueous phase was quantitatively measured over time using a standard curve established with a known amount of PGPE-BODIPY (Fig. 3A and B (open circles)). The production of palmitic acid was concurrently determined by TLC (Fig. 3B (closed circles)). A comparison of the formation of the BODIPY product with the release of palmitic acid showed that both products were formed at almost the same rate (Fig. 3B). This indicates that the BODIPY product found in the aqueous phase is 1-lyso-PGPE-BODIPY and that the hydrolysis reaction of PGPE-BODIPY by LPLA2 under acidic conditions is an *sn*-1 specific reaction. Thus, the formation rate of 1-lyso-PGPE-BODIPY directly reflects the activity of LPLA2 against PGPE-BODIPY. Fluorescence measurements of the aqueous phase were subsequently used to evaluate the present LPLA2 assay method using PGPE-BODIPY.

During the initial stage of the reaction, the formation of 1-lyso-PGPE-BODIPY increased linearly with time (Fig. 3C). As measured by the initial velocity in the reaction, LPLA2 activity was increased proportionally in a concentration dependent manner of the LPLA2 up to 100 μg/ml LPLA2 (Fig. 3D). The *Km* and *Vmax* values of the recombinant LPLA2 for PGPE-BODIPY were calculated as 5.64 μM and 20.7 μmol/min/mg protein, respectively (Fig. 4). Thus, the use of PGPE-BODIPY as a substrate provides a quantitative measure of LPLA2 activity.

Fluorescence measurement of LPLA2 activity of mouse serum using PGPE-BODIPY

To confirm whether the present method is specific for LPLA2 activity, sera obtained from wild type and LPLA2 deficient mice were used. The serum from wild type mouse catalyzed the formation of 1-lyso-PGPE-BODIPY under acidic reaction conditions but gradually diminished with time (Fig. 5A). By contrast, the serum from LPLA2-deficient mouse showed significantly lower catalytic activity than that from wild type mouse (Fig. 5A), although the LPLA2-deficient serum still possessed a weak but measurable activity toward PGPE-BODIPY. The remaining activity in the serum of LPLA2-deficient mouse was thought to be due to non-specific esterase or phospholipase activities. To improve the specificity of the reaction, the serum was pre-treated with an irreversible serine hydrolase inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), which is structurally similar to PMSF. As expected from insensitivity of LPLA2 to PMSF [9], AEBSF did not affect the enzymatic activity of LPLA2 (data not shown). However, the serum from LPLA2 deficient mouse pre-treated with 1 mM AEBSF completely lost the catalytic activity toward PGPE-BODIPY (Fig. 5B). By contrast, the serum from wild type mouse pre-treated with AEBSF not only maintained the catalytic activity against PGPE-BODIPY but also improved the reaction rate over the incubation time (Fig. 5B). These results show that pre-treatment of the serum with AEBSF is an effective means for reducing background in specific measurement of LPLA2 activity when using PGPE-BODIPY.

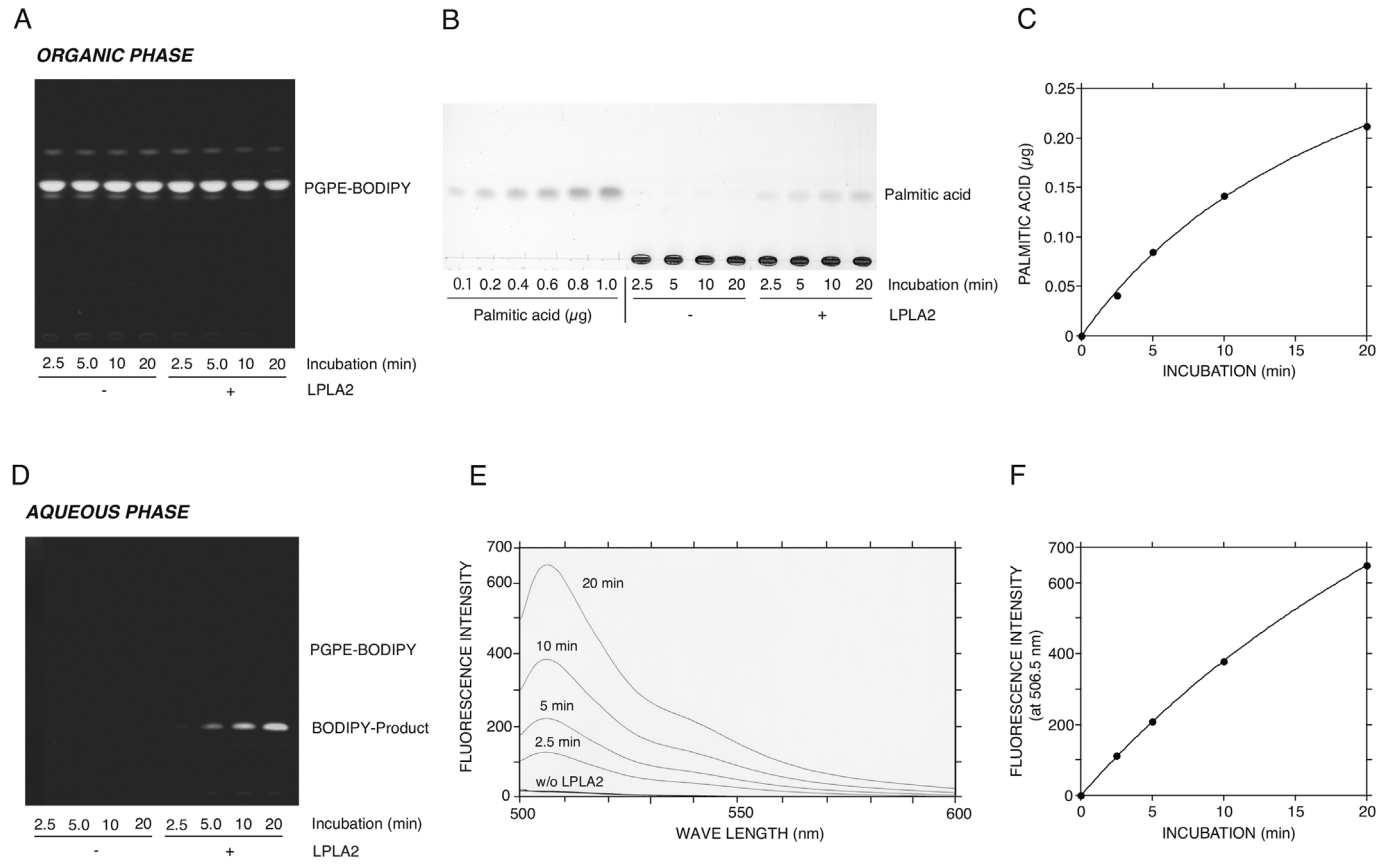

Fig. 2. Degradation of PGPE-BODIPY by LPLA2. The reaction was carried out as described in the Materials and Methods. PGPE-BODIPY/DODPC liposomes were incubated with or without 40 ng/ml recombinant mouse LPLA2 for 2.5, 5, 10 and 20 min at 37 °C in 500 μl of total volume. The reaction products recovered in the organic phase were separated using an HPTLC plate and developed in a solvent system consisting of chloroform/methanol/water (60:35:8 (v/v), **A**) or chloroform/methanol/pyridine (98:2:0.5 (v/v), **B**). To visualize BODIPY fluorophore, plate A was exposed under UV light. Plate B was charred as described in the Materials and Methods. For A, 20 μl of the organic phase was used. The remaining sample was applied to the plate B in parallel with different known amounts of palmitic acids. The production of palmitic acid was estimated by using a standard curve obtained from those palmitic acids (**C**). The reaction products recovered in the aqueous phase (50 μl) were separated with an HPTLC plate using a solvent system consisting of chloroform/methanol/water (60:35:8, v/v) and visualized under UV light (**D**). The fluorescence measurement of the aqueous phase was carried out as described in the Materials and Methods (**E**). Fluorescence intensity of BODIPY was plotted against the incubation time (**F**).

The formation of 1-lyso-PGPE-BODIPY proportionally increased in a concentration dependent manner as a function of the amount of AEBSF-treated wild type mouse serum assayed up until the concentration of serum reached 1% in the reaction mixture. When serum concentration was greater than 1%, 1-lyso-PGPE-BODIPY formation rate plateaued (Fig. 5C). However, the activity was measurable at the concentrations as low as 0.06% of serum in the reaction mixture by this method (Fig. 5D).

Discussion

Previously, we reported that a fluogenic phospholipid, 1-FAM-2-DABCYL-PG, can be used as a substrate for recombinant mouse LPLA2 [7]. However, we also observed that 1-FAM-2-DABCYL-PG was degraded by the serum obtained from LPLA2 deficient mouse under acidic conditions if 1-FAM-2-DABCYL-PG was incorporated into 1,2-dioleoyl-PC/sulfatide liposomes. In addition, the measurement of fluorescent emission of the reaction product formed from 1-FAM-2-DABCYL-PG by LPLA2 required the adjustment to a neutral or basic pH. In the present study, we attempted to develop a more convenient assay method to detect LPLA2 activity in various biological specimens.

A truncated oxidized phosphatidylethanolamine labeled with a BODIPY-fluoropher at the amino-group (PGPE-BODIPY, Fig. 1) was chosen as a substrate of LPLA2 for the following reasons. First, LPLA2 has a broad preference for the polar group of phospholipids [5]. Second, LPLA2 possesses a preferential selectivity and deacylase activity toward truncated oxidized phospholipids [8]. Third, PGPE-BODIPY is commercially available. Finally, unlike FAM-group, the fluorescence emission of BODIPY-group can be measured independent of pH.

In the present assay, the PGPE-BODIPY incorporated into DODPC liposomes at a molar ratio of PGPE-BODIPY to DODPC of 1–2.4 was self-quenching under acidic conditions. Therefore, the reaction solution showed red orange in color without fluorescence emission in the absence of the enzyme (Figs. 1B-2, left tube) but fluorogenic and light green with the addition of recombinant LPLA2 (Figs. 1B-2, right tube). However, when the liposomes consisted of the molar ratio of 1–24 for PGPE-BODIPY/DODPC, the solution was fluorogenic (data not shown), indicating that the increase in monomer emission of PGPE-BODIPY was evoked due to surface dilution by the additional DODPC. Thus, the quenching is probably due to self-aggregation of PGPE-BODIPY molecules in the liposomes.

The fluorescence emission of light green by the addition of LPLA2 is due to the monomeric emission of BODIPY-product, suggesting that the BODIPY-product formed by LPLA2 is a very polar molecule and is readily released from the liposome membrane into aqueous solution as a monomer. Accordingly, the direct measurement of the fluorescence of the reaction mixture may render a quantitative assay of LPLA2 activity possible.

As shown in our previous LPLA2 assay using 1-FAM-2-DABCYL-PG,

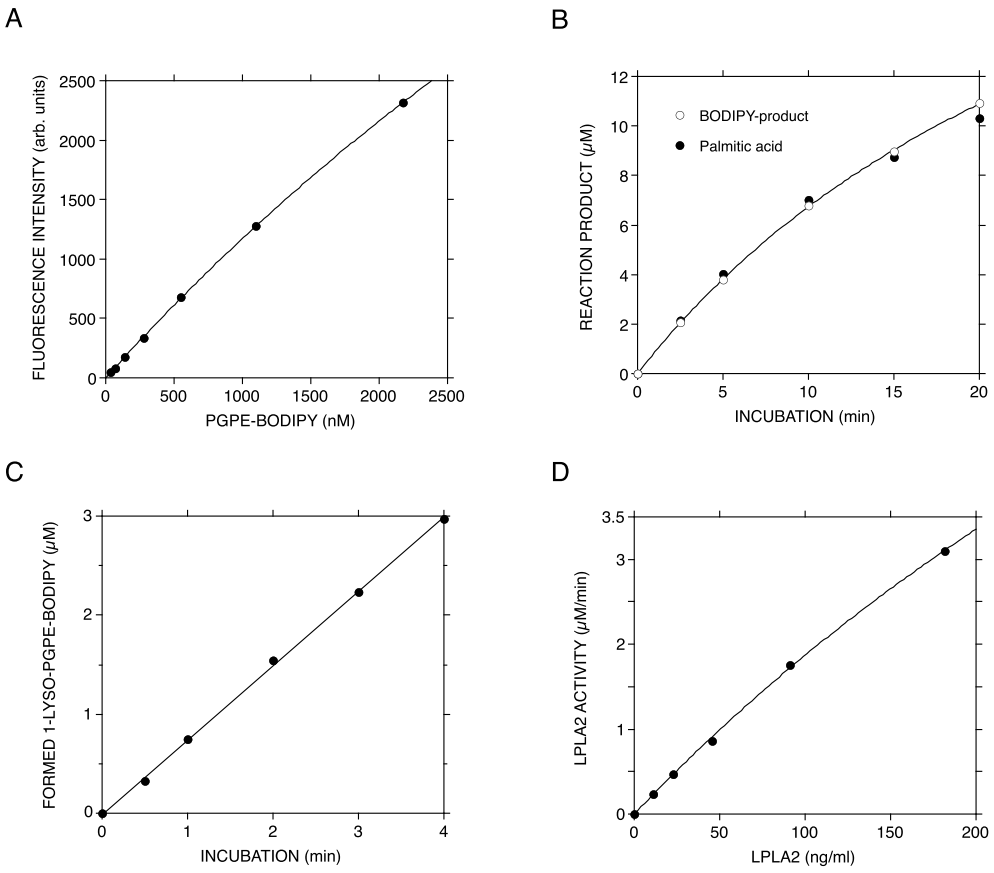

Fig. 3. LPLA2 activity of recombinant mouse LPLA2 on PGPE-BODIPY. In **A**, a standard curve of BODIPY in the aqueous phase was created using a known amount of PGPE-BODIPY. In **B**, the time course study was carried out as shown in Fig. 2. The reaction products, palmitic acid and BODIPY-product, were quantitatively measured by TLC and fluorescence measurement (**B**). Both product values represent their concentrations in the reaction mixture at each time point. In **C**, as shown in Fig. 2, PGPE-BODIPY/DODPC liposomes were incubated with or without 40 ng/ml recombinant mouse LPLA2 0.5, 1, 2, 3 and 4 min at 37 °C. After phase partitioning, the fluorescence of the aqueous phase was measured as described in the Materials and Methods. The 1-lyso-PGPE-BODIPY formed was plotted against the incubation time (**C**). In **D**, different concentrations of the LPLA2 were incubated as shown in Fig. C. The initial velocity of LPLA2 activity was plotted against the concentrations of LPLA2 in the reaction mixture (**D**).

a polar fluorogenic product, 1-FAM-lyso-PG, was isolated into the aqueous phase by phase partitioning using chloroform and methanol under acidic conditions although it was required to adjust a pH greater than 7 to gain the fluorescence emission of FAM [5]. Based on the same phase partitioning of the reaction mixture, BODIPY-PGPE was recovered in the organic phase (Figs. 1B-3, left). By contrast, the fluorogenic product formed from BODIPY-PGPE by LPLA2 was isolated from the substrate and freely dispersed into the aqueous phase, which provided the monomer emission of BODIPY without a pH adjustment (Figs. 1B-3, right). In this manner, the conventional lipid extraction method by phase partitioning (Figs. 1B-3) is suitable for the measurement of a large number of samples and of crude biological specimens.

One of the reaction products recovered in the organic phase after phase partition of the reaction mixture was identified as palmitic acid (Fig. 2B and C). This finding suggested that the other reaction product is 1-lyso-PGPE-BODIPY, which is structurally quite polar and easily distributed into aqueous phase as the free monomer. As expected, the fluorogenic reaction product was recovered in the aqueous phase after phase partition of the reaction mixture. This product showed a slower mobility in TLC analysis than PGPE-BODIPY (Fig. 2D) and was an alkaline unstable molecule. 2-Lyso-PGPE-BODIPY was recovered in the organic phase and had a faster mobility in the same TLC analysis than PGPE-BODIPY (data not shown). Therefore, the alkaline unstable molecule found in the aqueous phase was determined to be 1-lyso-PGPE-BODIPY.

As shown in Fig. 3B, the quantitative analyses for palmitic acid release and for BODIPY-product formation provided reliable stoichiometry for the hydrolysis reaction of PGPE-BODIPY by LPLA2. LPLA2 has the *sn*-1 preferential selectivity to the present fluorogenic truncated oxidized phospholipid as observed for natural truncated oxidized

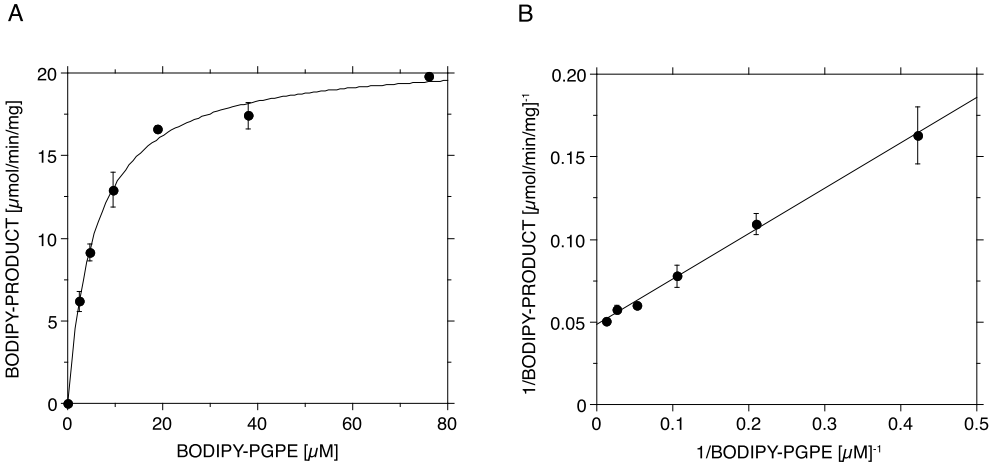

Fig. 4. *Km and Vmax* values of LPLA2 for PGPE-BODIPY. The initial velocities of LPLA2 activity at different concentrations of PGPE-BODIPY were determined as shown in Fig. 3 and were plotted against the concentrations of PGPE-BODIPY (**A**). **B** represents a reciprocal plot of figure A. Error bars indicate standard deviation (n = 3). The values of *Km and Vmax* of LPLA2 for PGPE-BODIPY were determined as 5.64 μM and 20.7 μmol/min/mg protein, respectively.

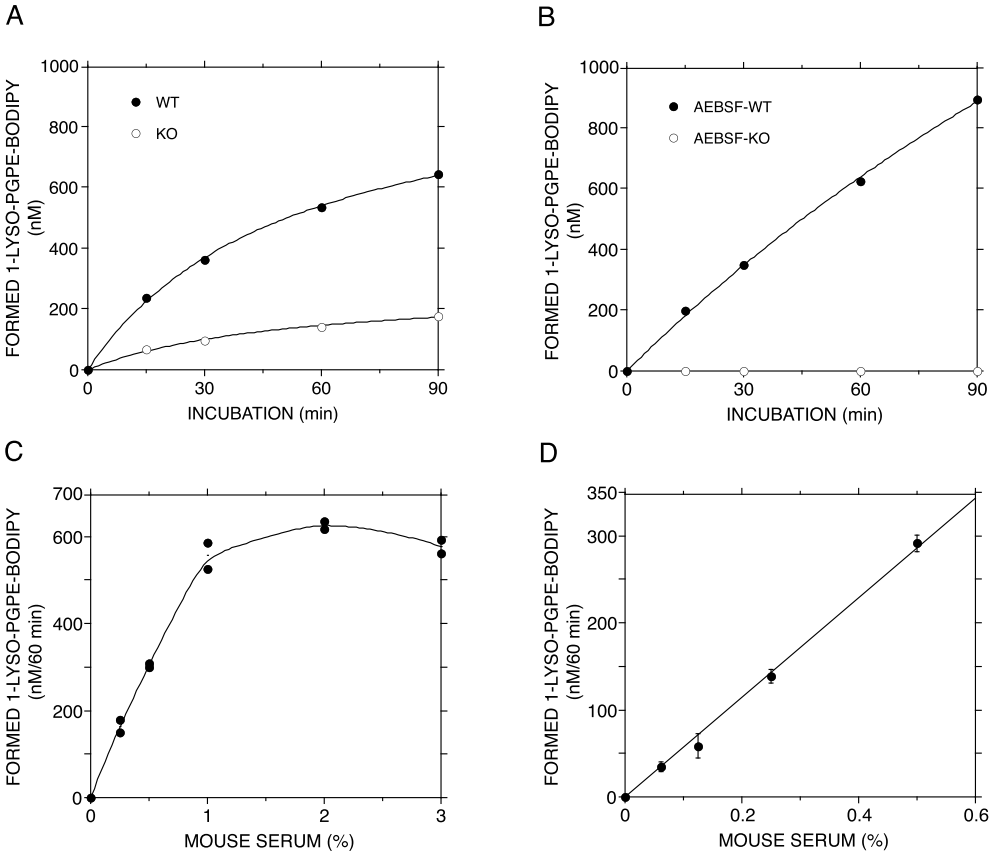

Fig. 5. Deacylation activity of PGPE-BODIPY in WT and LPLA2-deficient mouse sera. As shown in Fig. 2, liposomes consisting of PGPE-BODIPY/DODPC were incubated with or without 2% serum obtained from WT mice or LPLA2 deficient mice in 500 µl of total for 15, 30, 60 and 90 min at 37 °C. After phase partitioning, the reaction product, 1-lyso-PGPE-BODIPY, recovered in the aqueous phase was quantitatively measured as shown in Fig. 3 and plotted against incubation time (**A**). In **B**, the serum pre-treated with 1 mM AEBSF for 30 min at 37 °C was used. WT and KO denote the serum obtained from wild-type mice and from LPLA2 deficient mice, respectively. In **C** and **D**, different amounts of wild-type mouse serum pre-treated with 1 mM AFBSF were incubated with PGPE-BODIPY/DODPC liposomes for 60 min at 37 °C. The reaction product, 1-lyso-PGPE-BODIPY, recovered in the aqueous phase was quantitatively measured as described above. The rate of 1-lyso-PGPE-BODIPY formed in the reaction mixture was represented as the unit of nM/60 min and was plotted against the serum concentration in the reaction mixture. In **D**, error bars indicate standard deviation (n = 3).

phospholipids [8].

The reaction formula is shown as the following:

PGPE-BODIPY → 1-lyso-PGPE-BODIPY + palmitic acid

The kinetic constants, *Km* and *Vmax,* of LPLA2 for PGPE-BODIPY were also measured. The values of *Km* and *Vmax* were 5.64 µM and 20.7 µmol/min/mg protein, respectively (Fig. 4), which are similar to those of LPLA2 for dioleoyl phosphatidylglycerol (DOPG) [5]. DOPG is used to specifically detect the transacylase activity of LPLA2 in various biological specimens, but product analysis requires a classical TLC separation of products [6,10].

To see whether the present assay method is specific and suitable for biological specimens, the sera from wild type mice and LPLA2-deficient mice were used. The serum from wild type mice showed much higher deacylase activity than the serum from LPLA2-deficient mice (Fig. 5A). Although there was a weak deacylase activity at the *sn*-1 position of PGPE-BODIPY in the KO mouse serum, such non-specific activity was completely suppressed by pre-treatment of the serum with serine hydrolase inhibitor, AEBSF, of the serum (Fig. 5B). Like another structurally similar serine hydrolase inhibitor, PMSF, AEBSF did not affect recombinant mouse LPLA2 activity (data not shown). This treatment not only made the specific detection of LPLA2 activity in mouse serum possible but also improved the formation rate of 1-lyso-PGPE-BODIPY (Fig. 5B).

The serum contains PAF-acetyl hydrolase (PAF-AH), an enzyme that degrades truncated oxidized phospholipids in serum and is associated with LDL [11]. PAF-AH cleaves an oxidized truncated acyl group at the *sn*-2 position of oxidized phospholipids. PAF-AH activity is completely inhibited by AEBSF under the conditions employed here [12]. Using TLC analysis, we verified that the formation of 2-lyso-PGPE-BODIPY by the mouse serum pre-treated with 1 mM AEBSF was completely inhibited (data not shown). Thus, suppression of the consumption of PGPE-BODIPY by PAF-AH in the reaction mixture might partly account for the improvement in the formation rate of 1-lyso-PGPE-BODIPY by the LPLA2 in the serum.

Using the wild type mouse serum pre-treated with AEBSF, the formation of 1-lyso-PGPE-BODIPY increased linearly as a function of the serum concentration up to 1% (Fig. 5C). In addition, the activity was still reliably measurable using as little as 0.06% serum in the reaction mixture (Fig. 5D). Based on Fig. 5D, the LPLA2 activity of mouse serum is estimated as 57.2 pmol/60 min/µl of serum. Under the same assay condition, the specific activity of purified recombinant mouse LPLA2 showed 17.4 ± 0.777 µmol/min/mg protein (Fig. 4A), indicating that the mouse serum may possibly contain 1.274 nM of LPLA2. The LPLA2 activity reached a plateau in the presence of more than 1% of serum. We speculate that the activity is affected by the formation of insoluble materials observed when high concentrations of serum are used. A similar finding characterizes the transacylase assay using DOPG/DODPC liposomes [5]. The present fluorescence measurement could also be carried out by a plate reader equipped with fluorescence monitor. This would be a suitable means for the measurement of a large number of samples. On the contrary, the reliable measurement of LPLA2 activity in the serum by the method using DOPG as a substrate requires 1–2% serum in the reaction mixture and production separation by TLC [5].

Finally, the LPLA2 activity in liver extracts prepared from WT and LPLA2-KO mice was measured using the PGPE-BODIPY based method. It showed that the present assay is applicable to specifically determining the LPLA2 activity of the mouse liver extracts without loss of LPLA2 activity if using the extracts pre-treated with 10 mM AEBSF (data not shown).

Conclusions

PGPE-BODIPY is a sensitive and convenient reagent for the detection of LPLA2 activity. AEBSF can suppress non-specific deacylase activity toward PGPE-BODIPY without the loss of LPLA2 activity in mouse

serum and liver extracts. Therefore, the present assay method using both reagents is useful for the evaluation and screening of LPLA2 activity in a variety of biological specimens.

Conflicts of interest

The authors declare that there is no conflict of interest.

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