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Association with lipids or detergents is essential for preservation of the active structure of lipoprotein-associated phospholipase A_2

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ABSTRACT

Recombinant lipoprotein-associated phospholipase A_2 (rLp-PLA_2) expressed in HEK293 cells has a propensity to form oligomers in the absence of detergents. Dilution of rLp-PLA_2 in the absence of detergents results in irreversible inactivation of the enzyme. The monomeric rLp-PLA_2 may expose its hydrophobic interfacial binding region or substrate binding compartment to water and that may cause structural collapsing of the enzyme. Formation of self-aggregate, complex with binding partners or association with detergent micelles is to block the access of aqueous solvent to the hydrophobic substrate binding site and therefore prevents the structural collapsing. Dilution inactivation of the enzyme can be prevented in the presence of LDL or HDL suggesting that Lp-PLA_2 association with lipoprotein particles (LDL and HDL) is necessary for Lp-PLA_2 to maintain its enzymatic activity in human plasma. Formation of higher affinity complex gave better protection of rLp-PLA_2 structure and activity. The method can be harnessed to detect the interaction between rLp-PLA_2 and components of lipoprotein particles. Apo(a), ApoB 100 and ApoA1 were found to protect the enzyme from inactivation at roughly the similar level (~80 ± 5%) comparing to human serum albumin control (~40%). One mg/ml pig brain phospholipid showed 100% protection under the same conditions.

1. Introduction

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a Ca^{2+} independent plasma group VII phospholipase (Lp-PLA_2G7) bearing the structure similarity with other members of the phospholipase superfamily (Asano et al., 1999; Stafforini, 2009; Burke and Dennis, 2009; Tjoelker et al., 1995a; Samanta and Bahnson, 2008; Samanta et al., 2009). The pathological roles of Lp-PLA_2 in cardiovascular diseases (CVD) are presumably attributed to the generation of inflammatory hydrolysis products, lysophosphatidyl cholines and oxidized free fatty acids (Tjoelker et al., 1995b). The majority of the circulating human Lp-PLA_2 in blood is synthesized by macrophages and the matured enzyme is a 45–50 kD glycosylated protein (Stafforini et al., 1990). Normally, the secreted enzyme in the plasma has been shown to associate with high density lipoproteins (HDL) and low density lipoproteins (LDL) in the ratio of about 1:2 (Gardner et al., 2008; Zhuo et al., 2017). Clinical studies have suggested that the pathogenicity of Lp-PLA_2 may be affected by the pattern of lipoprotein affiliation (Karabina et al., 1997; Zhuo and Yuan, 2018). It was reported that the ratio of Lp-PLA_2 in lipoproteins may affect the enzymatic activity and determine its physiopathological functions in humans (Stafforini et al., 1989; Tsimihodimos et al., 2002). However, a potent Lp-PLA_2 inhibitor, darapladib, failed in two pivotal trials to meet both primary and secondary endpoints (White et al., 2014; O'Donoghue et al., 2014). It raises a question whether Lp-PLA_2 may play roles in CVD pathology. Nevertheless, Lp-PLA_2 remains an effective diagnostic biomarker for CVD and its association with lipoproteins may be critical for its functions (Zhuo and Yuan, 2018). Human plasma Lp-PLA_2 are mainly produced by hematopoietic and hepatic cells. However, the mechanism to associate with LDL and HDL are not well studied and the interaction of Lp-PLA_2 with some

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; DMPC, 1,2-dimyristoyl-sn-glycerol-3-phosphocholine; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGS, ethylene glycol bis[succinimidyl]succinate; ELISA, Enzyme-Linked Immuno Sorbent Assay; FBS, fetal bovine serum; HDL, high density lipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDL, low density lipoprotein; MES, 4-Morpholineethanesulfonic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNS, sodium 1-nonane sulfonate; TBS, tris buffered saline; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TMB, 3.3', 5.5'-tetramethylbenzidine; Tris, tris(hydroxymethyl)aminomethane

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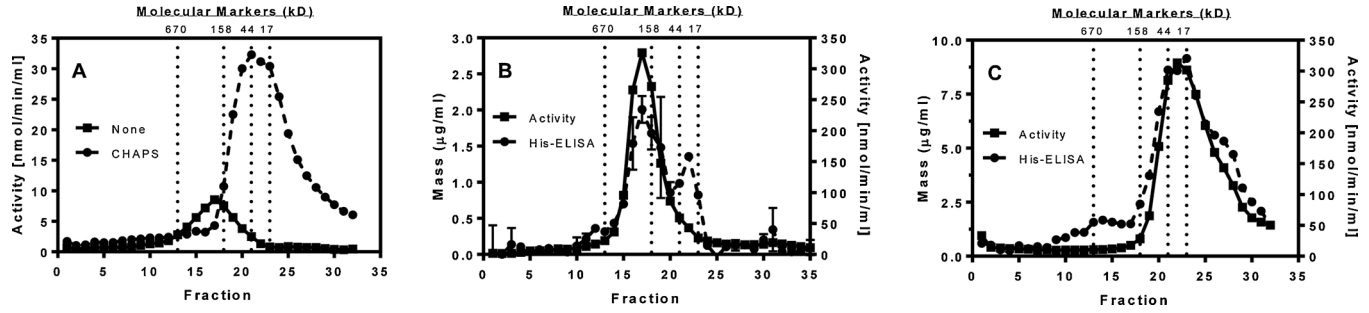

Fig. 1. Fractionation of rLp-PLA$_2$ with Superose-6 column. **A.** Fractionation of rLp-PLA$_2$ with Superose-6 column in the presence and absence of 10 mM CHAPS. Ten µl of purified rLp-PLA$_2$ in 50 mM Tris/HCl, pH 8.0, containing 5 mM CHAPS at the concentration of 1.1 mg/ml were fractionated by a 10 cm x 300 cm Sperose-6 column equilibrated in 50 mM sodium phosphate, pH 7.4, containing 100 mM sodium chloride, 2 mM EDTA and 0.02% sodium azide. Fraction were collected at 0.6 ml/tube with 0.3 ml/min flowrate. Five µl from each fraction were assayed PLAC® activity as described in the experimental section. **B.** Fractionation of C-terminal His-tag rLp-PLA$_2$ with Superose-6 column in the absence of detergents. Eleven µl of purified rLp-PLA$_2$ with a C-terminal His-tag at 2.96 mg/ml were fractionated in the same process as in A. Forty five µl from each collected fraction were assayed by PLAC® activity and fifty µl were assayed by HisGrap-ELISA. **C.** Fractionation of purified C-terminal His-tag rLp-PLA$_2$ with Superose-6 column in the presence of 10 mM CHAPS. Fifteen µl of rLp-PLA$_2$ with a C-terminal His-tag at 0.7 mg/ml were fractionated in the same process as in **A**. Fifty µl from each collected fraction were assayed by PLAC® activity and fifty µl were assayed by HisGrap-ELISA.

components of LDL and HDL is not conclusive (Stafforini et al., 1989; Srinivasan and Bahnson, 2010). We sought to use the recombinant Lp-PLA$_2$ (rLp-PLA$_2$) expressed in HEK293 to study the mechanism of interaction between the enzyme and lipoprotein lipids.

2. Results

2.1. Association of Lp-PLA$_2$ with detergent micelles

To estimate the molecular size of the rLp-PLA$_2$ expressed in HEK293 cells, the purified enzyme was subjected to fractionation by a 10 × 300 mm Superose-6 column in the presence and absence of 10 mM CHAPS. The results indicated that the same enzyme was eluted very differently under the various conditions (Fig. 1A). According to the molecular weight reference, rLp-PLA$_2$ was eluted between the chicken ovalbumin (44 kD) and horse myoglobin (17 kD) in the presence of 10 mM CHAPS and between the bovine thyroglobulin (670 kD) and bovine Ig-globulin (158 kD) in absence of the detergent (chromatography of molecular markers not shown). The expected molecular weight of rLp-PLA$_2$, not including the glycosylation oligosaccharide chains, is about 48 kD. To further understand the retention time shift, we resolved the enzyme by the same procedure with different detergents. The results showed that the column retained rLp-PLA$_2$ differently with different detergents (Table I). Detergents with larger micelle molecular weight eluted rLp-PLA$_2$ earlier from the column. This indicates the association of rLp-PLA$_2$ with the micelles of the detergents. However, the molecular size of the rLp-PLA$_2$ in the absence of the detergents seems larger than that of the complex of enzyme and detergent micelles tested. This suggests a possibility that the enzyme may form oligomeric structures or aggregate in the absence of detergents. We also fractionated the unpurified rLp-PLA$_2$ from the cell cultural supernatant of HEK293 and it gave the same results as the purified enzyme under the same conditions (results not shown). Another observation was that the recovery yield based on the PLAC® activity enzymatic assay was much lower when rLp-PLA$_2$ was fractionated in the absence of detergents (Table 1). In the absence of detergents, only about 23% of rLp-PLA$_2$ activities were recovered compared to 60–146% recovery in the presence of detergents. To investigate the lost rLp-PLA$_2$ in the absence of detergents, purified rLp-PLA$_2$ with a His-tag at the C-terminal was subjected to fractionation and the fractions were assayed by both the PLAC® activity assay and the His-ELISA using rabbit anti-Lp-PLA$_2$ polyclonal antibody. When rLp-PLA$_2$ was fractionated in the absence of detergents, two mass peaks (fraction 16–18 and 21–23) were shown by the His-ELISA but only one activity peak (fraction 16–18) was seen by the PLAC® activity assay (Fig. 1B). That is, the lower molecular weight mass peak (fraction 21–23) contained no enzymatic activity. However, when the enzyme was fractionated in the presence of 10 mM CHAPS in the same buffer, no mass or enzymatic activity at fraction 16–18 was seen but both mass and enzymatic activity were detected at the fraction 21–23 (Fig. 1C). This suggests that the lower molecular weight peak (fraction 21–23) losses its activity irreversibly in the absence of detergents. In the presence of detergents, rLp-PLA$_2$ is probably stabilized by the formation of the complex with detergent micelles.

2.2. Inactivation of rLp-PLA$_2$ by dilution in the absence of detergents

It was found that freshly prepared rLp-PLA$_2$ stored in the presence or absence of detergents had no difference in specific activity when assayed with PLAC® activity assay (results not shown). However, the enzyme stored in the absence of detergents at 4 °C lost its activity faster, especially when the concentration was low (results not shown). To further investigate the decrease of rLp-PLA$_2$ specific activity in the absence of detergents, the enzyme was subjected to dilution to the final concentration between 1–3 µg/ml in PBS, pH 7.2, and the changes of the enzymatic activity and immuno-reactive mass were followed. The immuno-reactive mass of Lp-PLA$_2$ was quantified by using the PLAC® mass assay that only recognized the non-denatured form of the enzyme (conformational). Fig. 2 shows that the enzyme gradually lost its activity and immuno-reactive mass in two phases. Upon dilution, the enzymatic activity and the immuno-reactive mass had a sharp decline phase (about 1–2 days of incubation at 4 °C) and then the inactivation rate decreased and transferred to a slower phase (Fig. 2). The final normalized losses in both activity and immuno-reactive mass were in the range of 50–75% at the fifteenth day of incubation.

2.3. The effects of detergents on the activity of rLp-PLA$_2$

The effects of detergents on the dilution inactivation of rLp-PLA$_2$ were investigated. When 10 mM CHAPS was included in the dilution buffer, no inactivation was observed for the diluted rLp-PLA$_2$ at 1 µg/ml (Fig. 3A). However, the addition of 10 mM CHAPS into the inactivated enzymes only recovered a very small portion of the lost activity but it did prevent the enzyme from further inactivation during the extended incubation (Fig. 3A). In addition to CHAPS, several other non-ionic detergents, such as Tween-20, Triton X-100 and digitonin, were also found protective in the dilution inactivation of rLp-PLA$_2$ (data not shown). Detergents with high CMC were less effective than those with lower CMC. In an experiment of dilution inactivation for rLp-PLA$_2$, the diluted enzyme was incubated in buffers containing variable detergent concentrations from 0.15 mM to 10 mM. The rate of enzyme inactivation was found to be concentration dependent for CHAPS

Table 1
Retention time and recovery yield of rLp-PLA_2 in different detergents.

Detergent	Activity Recovery Yield (%)	Retention (Peak Fraction)	CMC (mM)	Micelle MW (kD)	Reference
None	23.4	16			
10 mM CHAPS	72.4	21	6-10	6	(Menzies et al., 1999; Bhairi, 2001)
10 mM Deoxycholate	60.4	21	1.5-6	8	(Menzies et al., 1999; Bhairi, 2001; Jover et al., 1997)
5 mM Digitonin	91.5	18	0.09	74	(Menzies et al., 1999; Bhairi, 2001)
1% Tween-20	111.4	18	0.06	76	(Menzies et al., 1999; Bhairi, 2001)
1% Triton X-100	146.8	18	0.3	90	(Menzies et al., 1999; Bhairi, 2001)

Purified rLp-PLA_2 was fractionated using a newly purchased Superose-6 column and 5–25 µl of each fraction were assayed by PLAC® activity kit as described in the experimental section. Peak fractions were used to represent the retention time. Yields were calculated via dividing the total activity units from all fractions by the total activity units injected. Over recovery of Triton X-100 was due to the high background of the detergent in PLAC® activity assay.

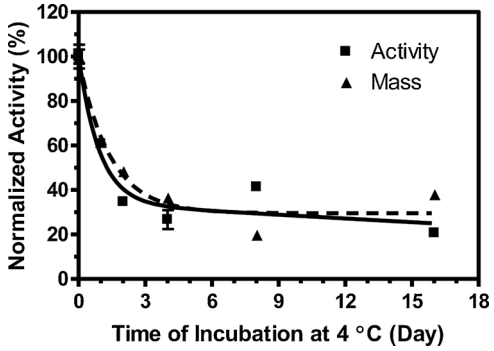

Fig. 2. Purified rLp-PLA_2 was diluted into PBS, pH 7.2, at the final concentration of 2.9 µg/ml and the solution was incubated at 4 °C. Five µl of the solution was drawn at the indicated time for the activity assay by PLAC® activity test. One µl was further diluted 50 fold and 20 µl of the diluted solution were assayed for mass by PLAC® mass test. All mass and activity values were normalized against the initial value.

(CMC = 6 mM) and deoxycholate (CMC = 1.5 mM) but not for Triton X-100 (CMC = 0.3 mM), Digitonin (CMC = 0.09 mM) and Tween-20 (CMC = 0.06 mM) (Fig. 3B). This suggests that detergent micelles, instead of monomeric detergent, are the stabilizer of rLp-PLA_2 molecule.

2.4. The effects of the protein concentration on the activity of rLp-PLA_2

At high concentrations (> 0.5 mg/ml), rLp-PLA_2 is fairly stable even in the absence of detergents (observation not shown). In the dilution inactivation of the recombinant Lp-PLA_2, the inactivation rates are dependent on the final diluted concentration of the enzyme. The concentration effect on the rLp-PLA_2 dilution inactivation is illustrated in Fig. 4A. The rate and final loss of the rLp-PLA_2 inactivation upon dilution varied in the enzyme concentration range of 0.6–5 µg/ml. The inactivation rates became relatively independent of final enzyme concentrations at both ends of the above concentration range. This can be better demonstrated by plotting the residual percentage of the rLp-PLA_2 activity after the enzyme was diluted and incubated at 4 °C for ten days against the protein concentrations (Fig. 4B). In the logistic scale of concentration, it can be fitted into a sigmoidal curve. There is a sensitive range between 1 and 5 µg/ml.

2.5. Protection of rLp-PLA_2 activity by lipoproteins

Lp-PLA_2 protein has been shown to associate with LDL and HDL in human plasma (Gardner et al., 2008; Zhuo et al., 2017). Experiments were designed to reveal if LDL and HDL would prevent rLp-PLA_2 from the inactivation during the dilution into non-detergent containing buffers. Purified rLp-PLA_2 was diluted in 50 mM sodium phosphate buffer, pH 7.2, containing 150 mM sodium chloride and 2 mM EDTA at the final concentration of 0.5 µg/ml enzyme and incubated at 4 °C for 2 days. The experiments were carried out in the presence of various

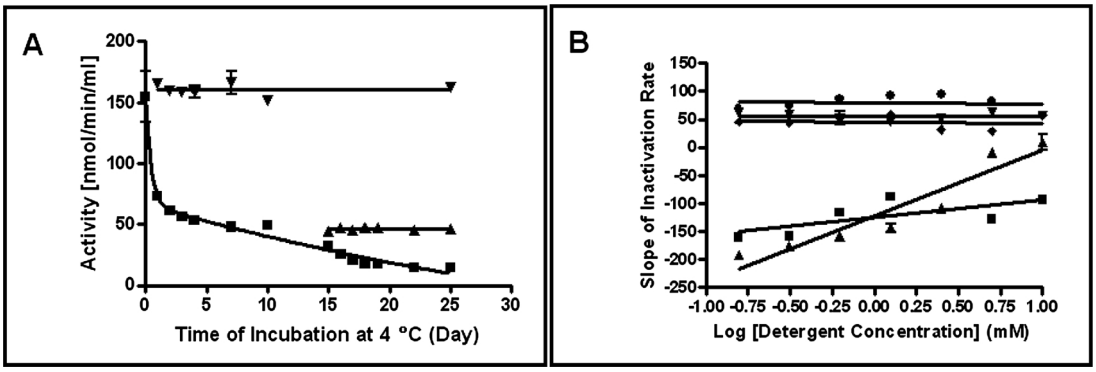

Fig. 3. Effects of detergents on the Dilution Inactivation and activity of rLp-PLA_2. **A.** Protection of rLp-PLA_2 from dilution inactivation by 10 mM CHAPS. Purified rLp-PLA2 in 50 mM Tris/HCl, pH 8.0 with 10 mM CHAPS was diluted 1000 fold into PBS, pH 7.5, with (▼) and without (■) 10 mM CHAPS at the final concentration of 1.3 µg/ml. Samples were incubated at 4 °C. At the indicated time point, 5 µl each sample were used to assay for activity by using PLAC® activity kit as described in the experimental section. At 15th day of the incubation, 100 µl of the enzyme mixture without detergent were withdrawn to mixed with 2 µl of 0.5 M CHAPS to obtain the final detergent concentration of 10 mM (▲). Enzymatic activities were monitored for another 10 days using the same method. **B.** Dose dependence of detergents in the protection of the rLp-PLA_2 from dilution inactivation. Purified rLp-PLA_2 in 50 mM Tris/HCl, pH 8.0 with 10 mM CHAPS was diluted 1340 fold at the final concentration of 1 µg/ml into 50 mM sodium phosphate, pH 7.0, containing 100 mM sodium chloride and various concentration of detergents: CHAPS (■), sodium deoxycholate (▲), triton X-100 (▼), Digitonin (♦) and Tween-20 (●). The mixtures were incubated at room temperature and the activities of the enzyme were assayed by PLAC® activity test as described in the experimental section. The initial inactivation rates were obtained by linear regression analyses of the rLp-PLA_2 inactivation within the incubation time from 0 to 500 min and plotted against the logistic values of detergent concentrations are presented as a Lineweaver-Burke plot.

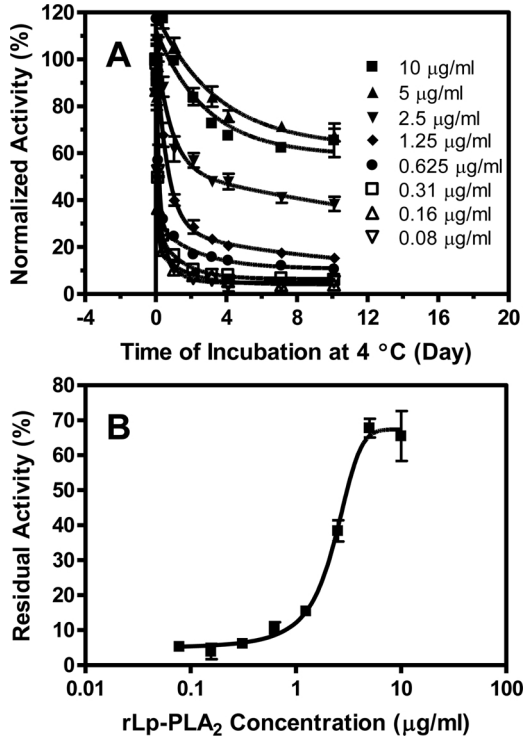

Fig. 4. Concentration effects on the dilution inactivation of rLp-PLA_2. **A.** rLp-PLA_2 in 50 mM Tris/HCl, pH 8.0, with 10 mM CHAPS was diluted into PBS, pH 7.2, at the indicated final concentrations. Activities were followed by PLAC® activity assay at the indicated time. Volumes used for assays were adjusted based on the concentration of the enzyme so that the determined activities were in the linear range. Activities were normalized to the initial values. Assay conditions were as described in the experimental section. **B.** The normalized activities on Day 10 were plotted against the final concentrations of rLp-PLA_2.

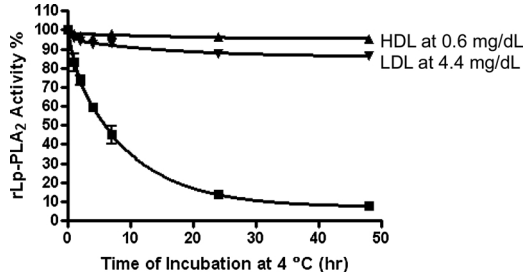

Fig. 5. Protection of rLp-PLA_2 from inactivation during dilution by HDL and LDL. Purified rLp-PLA_2 was diluted in 50 mM sodium phosphate buffer, pH 7.2, containing 150 mM sodium chloride and 2 mM EDTA at the final concentration of 0.5 μg/ml enzyme and incubated at 4 °C for 2 days. The experiments were carried out in the presence of various concentrations of fractionated LDL and HDL (devoid of endogenous Lp-PLA_2 activity). Only the selected data are presented and the lipoprotein concentrations are indicated as that of triglyceride.

concentrations of fractionated LDL and HDL (devoid of endogenous Lp-PLA_2 activity). It was indeed found that the dilution inactivation of rLp-PLA_2 could be averted in the presence of either LDL or HDL particles. Fig. 5 shows that human LDL or HDL at concentrations 4.4 and 0.6 mg/dl of triglyceride respectively fully protected the rLp-PLA_2 activity during the dilution in the phosphate buffer. No significant activity losses were observed after the two day period of incubation at 4 °C in the LDL or HDL containing buffer while more than 90% of the original activity vanished in the control buffer. Such protection effects can be extended even when LDL and HDL were reduced to as low as 1.4 and 0.14 mg/dl respectively (results not shown).

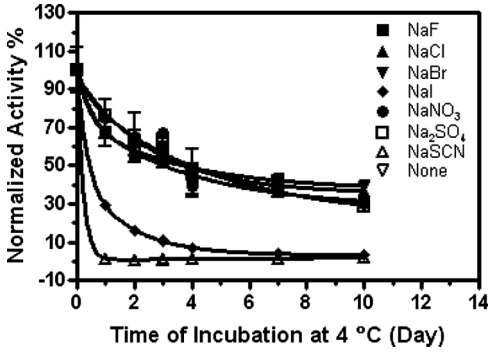

Fig. 6. Effects of chaotropic agents on the stability and activity of rLp-PLA_2. **A.** Effects of different anions on the dilution inactivation of rLp-PLA_2. Purified rLp-PLA_2 in 50 mM Tris, pH 8.0, with 10 mM CHAPS was diluted 1000 fold to the final concentration of 1.3 μg/ml in 12.5 mM sodium phosphate, pH 7.6, containing 1 M of the indicated salt. The enzyme mixtures were incubated at 4 °C and the activities were followed by PLAC® activity assay as described in the experimental section using 5 μl of the enzyme mixture. Data points were fitted with Boltzmann sigmoidal curves.

2.6. The effects of chaotropic agents on the activity of rLp-PLA_2

According to the gel permeation experiments, detergents could reduce the molecular weight of rLp-PLA_2 and stabilize its activity. To investigate the connection between the deoligomerization and stabilization effects of detergents, rLp-PLA_2 was diluted and incubated at 4 °C in the presence of 1 M sodium salts of fluoride, bromide, chloride, iodide, nitrate, sulfate (0.5 M) and thiocyanate. While detergents were found to stabilize rLp-PLA_2, anions destabilizing protein-protein interactions, such as $SCN^{-1 \text{ or } I^{-1}}$ (Zhang and Cremer, 2006), were found to promote the inactivation of the enzyme. The inactivation of the diluted rLp-PLA_2 during the incubation at 4 °C was significantly accelerated by including 1 M of NaSCN or NaI in the incubation buffer (Fig. 6). This is not due to the added sodium salt concentration because no other salts had effects on the stability of the enzyme. None of the above chemicals (up to 1 M) was found inhibitory to the enzymatic activity of rLp-PLA_2 either (results not shown).

2.7. Chemical cross-linking of rLp-PLA_2

To further confirm the formation of the oligomeric rLp-PLA_2 during dilution, the highly purified enzyme was diluted into buffers containing a chemical cross-linker, ethylene glycol bis[succinimidylsuccinate] (EGS), with and without detergents. Fig. 7 shows the results of the cross-linking experiment. First of all, when rLp-PLA_2 was diluted to the final concentration of 1 μg/ml in the absence of detergents, only oligomers with molecular weight > 98 kD were detected on the Western Blot by rabbit anti-Lp-PLA_2 antibody. No monomeric (48 kD) and only a small amount of dimeric (98 kD) rLp-PLA_2 were seen. These results prove that rLp-PLA_2 does quickly self-associate and form polymers upon dilution in the absence of lipid substrates or detergents. Second, the extent of rLp-PLA_2 oligomerization observed was different when stored at different conditions. Enzyme stored in buffer containing 5 mM CHAPS had a lower oligomerized molecular weight than enzyme stored in the detergent-free condition although both were diluted into the same cross-linking buffer at the same final concentration. Third, in the presence of 10 mM CHAPS (or 1% Tween-20, data not shown), the majority of rLp-PLA_2 stayed monomeric after cross-linked by EGS. Again, rLp-PLA_2 stored in the presence of 5 mM CHAPS was almost free of oligomeric bands when cross-linked in buffer containing detergents while that stored at detergent-free conditions still had significant amounts of high molecular weight species when cross-linked in the same buffer. These suggest even at high concentration, without detergents, rLp-PLA_2 may still form soluble aggregate and the size of the

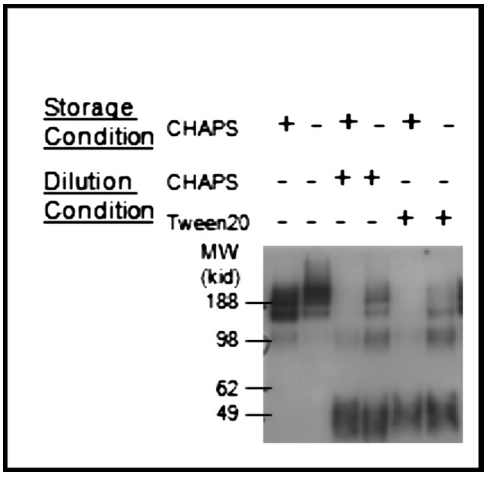

Fig. 7. Cross-linking of rLp-PLA$_2$. rLp-PLA$_2$ was stored and cross-linked under different conditions as indicated. The cross-linked proteins were then resolved by SDS-PAGE and signals were detected by Western analyses. Procedures are as described in the experimental section.

aggregate may grow over the storage time. The detergents do not reduce the reactivity of EGS in the cross-linking of rLp-PLA$_2$ because the yield of control experiments in internally cross-linking IgG by EGS was not affected by the presence of the same detergents (data not shown).

2.8. Protection of rLp-PLA$_2$ from inactivation by components of lipoprotein particles

The recombinant Lp-PLA$_2$ possibly interacts with components of LDL and HDL through the same mechanism as self-aggregation. Some components of LDL and HDL were incubated and screened for their interaction with rLp-PLA$_2$. Recombinant Lp-PLA$_2$ was diluted into phosphate buffer (50 mM sodium phosphate buffer, pH 7.2, containing 150 mM sodium chloride and 2 mM EDTA) containing each of the followings, 0.6 mg/ml Apo(a), 1 mg/ml ApoB100, 1 mg/ml ApoA1, 10 mg/ml human serum albumin (rHSA), 1 mg/ml suspension of triglyceride, mdt-glyceride (mix of mono-, di- and tri-glyceride), free cholesterol, cholesterol oleate or pig brain phospholipid, 5 mM digitonin, 1 M NaCl or NaSCN. The mixtures were incubated at 2–8 ∘C for 8 days and the residual rLp-PLA$_2$ was assessed by PLAC® mass assays (Fig. 8A). All complexes were assayed by PLAC® mass assay with and without 10 mM CHAPS in the assay buffer to avoid antibody binding bias due to rLp-PLA$_2$ interaction with binding partner or detergent micelles. Full protection was achieved by the incubation of rLp-PLA$_2$ in 5 mM digitonin and structural collapsing was induced by the incubation of rLp-PLA$_2$ in 1 M NaSCN, which prevents rLp-PLA$_2$ to form aggregate (self-oligomers) or complex with other partners. Apo(a), ApoB100, ApoA1 and phospholipid were found almost fully protecting rLp-PLA$_2$ from structural collapsing, indicated by at least one type of PLAC® mass assays. The diluted rLp-PLA$_2$ maintained some mass (about 40%–60% of original mass) in recombinant human serum albumin (rHSA), mdt-glyceride, cholesterol oleate ester, free cholesterol and NaCl. The enzyme only retained a small portion of mass when incubated in 1 mg/ml suspension of triglyceride. The diluted rLp-PLA$_2$ solutions were also monitored by PLAC® activity assay for its enzymatic activity while incubated at 2–8 ∘C (Fig. 8B). The results are similar with that of PLAC® mass in Fig. 8A. The added compounds can be devided as 4 groups. The first group is 5 mM digitonin and 1 mg/ml pig brain phospholipids which fully protected rLp-PLA$_2$ activity from loss at the 8th day of incubation. The second group includes 0.6 mg/ml Apo(a), 1 mg/ml ApoB100, 1 mg/ml ApoA1 and 1 mg/ml mdt-glyceride which maintained significant rLp-PLA$_2$ enzyme activity (≥70%) during the incubation. The third group composes 1 mg/ml each of tri-glyceride,

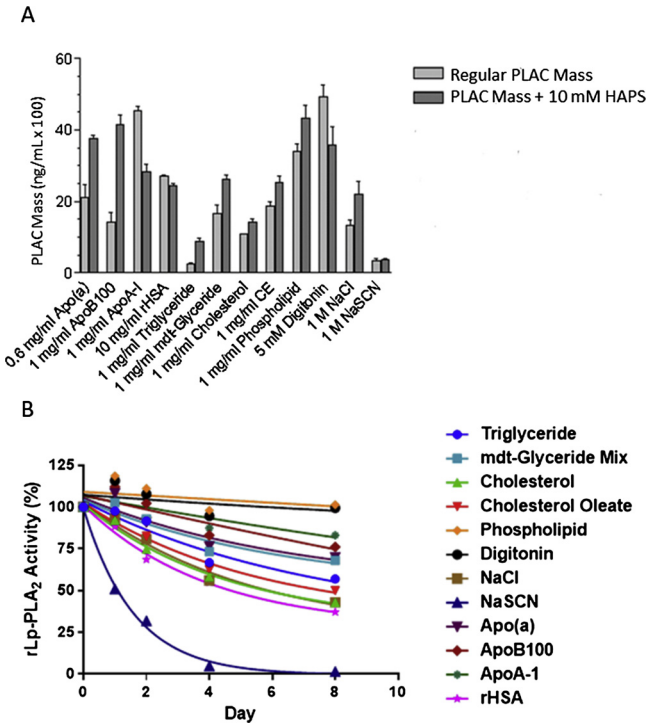

Fig. 8. Protection of rLp-PLA$_2$ from inactivation during dilution by some components of HDL and LDL. Purified rLp-PLA$_2$ was diluted in 50 mM sodium phosphate buffer, pH 7.2, containing 150 mM sodium chloride and 2 mM EDTA at the final concentration of 3.4 μg/ml enzyme and incubated at 2–8 ˚C for 8 days. The experiments were carried out in the presence of various concentrations of proteins or compounds as indicated. Fig. 8**A:** Samples were withdrawn to be assayed by PLAC® mass kit with and without 10 mM CHAPS at the end. Fig. 8**B:** Samples were withdrawn to be assayed by PLAC activity kit at the time indicated.

cholesterol oleate, free cholesterol, 1 M NaCl and 10 mg/ml rHSA. For the third group, rLp-PLA$_2$ enzyme activity was reduced to around 50% ± 10% of the starting level. Complete loss of Lp-PLA$_2$ enzyme activity was found in 1 M NaSCN solution at the 4th day of incubation.

3. Discussion

In the characterization of rLp-PLA$_2$ by size exclusion chromatography, it was found that the recovery yield in the absence of detergents was very low as shown by the PLAC® activity assay of the collected fractions (Table 1). By including 10 mM CHAPS in the chromatography buffers, not only the recovery yield was improved but also the molecular size of the rLp-PLA$_2$ was reduced. This suggests that the enzyme may not exist as the monomeric form in the absence of detergents. Indeed, fractionation of the C-terminal His-tag rLp-PLA$_2$ in the absence of detergents and assaying the fractions by HisGrap-ELISA using rabbit anti-Lp-PLA$_2$ polyclonal antibodies, which detects both the native and denatured rLp-PLA$_2$, we demonstrate that a lower molecular weight mass peak without enzymatic activity was missed by the PLAC® activity assay (Fig. 1). It is unlikely that the mass without activity comes from the impurity that cross reacts with the polyclonal antibodies because the recombinant protein has been purified to highly homogeneous purity and subjected to SDS-PAGE and Western Blotting analyses (data not shown). The results suggest that it is the monomeric rLp-PLA$_2$ that may not be stable in the absence of detergent and it can also be inferred that the enzyme may form oligomers in the absence of detergents in order to keep the hydrophobic sites away from aqueous solvent. When diluted, there will be more monomeric rLp-PLA$_2$ formed because of the increase in the rate for dissociation and the decrease in the rate for oligomer formation. Thus, dilution will cause more inactivation of the

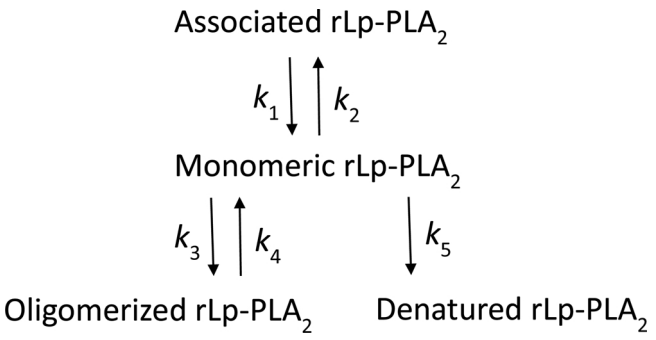

Fig. 9. The recombinant Lp-PLA_2 exists in reversible association each other in solution at concentration > 5 μg/mL. When it is diluted to lower concentration, Lp-PLA_2 dissociates to monomeric form which either forms oligomer or becomes denatured in solution as inactive monomer. The oligomer can slowly dissociate to monomer and become denatured. The oligomerization and deoligmerization are reversible steps but the denaturation is an irreversible step which leads the enzyme stock gradually lost activity.

enzyme in the absence of detergents. This is indeed the case observed in our study. To explain the dependence of the inactivation rate on the rLp-PLA_2 concentrations, a model is proposed (Fig. 9). At high protein concentration, the association between rLp-PLA_2 molecules is fast and the monomeric rLp-PLA_2 is less abundant and, therefore, the enzyme is stable. Dilution or breaking protein-protein interaction will increase monomeric rLp-PLA_2 and, in the absence of substrate or detergents, it will result in the inactivation of the enzyme. The instability of monomeric rLp-PLA_2 can also be demonstrated by the effects of chaotropic agents in the dilution of the enzyme. The experiment shows that the protein-protein interaction breaker such as SCN^{-1} or I^{-1} destabilizes rLp-PLA_2. It can be inferred that rLp-PLA_2 tends to form a dimer or oligomers during the dilution but, if the self-interaction is prevented or interrupted by chaotropic agents, the formed monomeric enzyme will be denatured, possibly due to exposure of the hydrophobic substrate binding site to aqueous solvents. Qualitative analysis indicates k1 and k2 > k3 and k5 > k4.

Lp-PLA_2 has been speculated to play different function when associated to different lipoprotein particles (Tellis and Tselepis, 2009). Understanding the binding partners of Lp-PLA_2 in different lipoproteins is important to reveal the regulation of Lp-PLA_2. Therefore, the above discovery of the dependence of Lp-PLA_2 stability on its association with other hydrophobic partners can be harnessed to compare the binding affinity among LDL/HDL components. When rLp-PLA_2 is diluted into solution containing potential binding partners or other hydrophobic materials, the formation of complexes between Lp-PLA_2 and these compounds is expected to protect the enzyme from structural collapsing. The higher affinity of the complex, the more stable is rLp-PLA_2. Fig. 8 shows the protection effects of some components of LDL or HDL. Apo(a), ApoB100 and ApoA1 at the final concentration of 0.6–1 mg/mL significantly preserved the rLp-PLA_2 native structure comparing to 10 mg/ml rHSA. Binding of Lp-PLA_2 with Apo(a) and ApoB100 has been demonstrated by other methods (Srinivasan and Bahnson, 2010). However, binding between Lp-PLA_2 and ApoA1 is never conclusively determined (Srinivasan and Bahnson, 2010). The data in Fig. 8 clearly supports the existence of interaction between rLp-PLA_2 and ApoA1. Fig. 8 also shows a strong protection of rLp-PLA_2 structure by pig brain phospholipid comparing to other LDL and HDL components such as triglyceride, mdt-glyceride (mix of mono-, di- and tri-glyceride), free cholesterol or cholesterol oleate ester at the same mass (1 mg/mL). These data suggest that although the Apo(a), ApoB100 and ApoA1 may have high binding affinity with Lp-PLA_2, the phospholipid monolayer is the main driving force to mediate the association of Lp-PLA_2 with Lp(a), LDL and HDL. Other hydrophobic components only play a minor role in Lp-PLA_2 binding. It can then be inferred that Lp-PLA_2 may prefer to stay on the phospholipid monolayer instead to be buried inside the lipoproteins under the incubation conditions (2–8 ∘C). Indeed, it has been observed that human sera incubated at 4 ∘C have higher PLAC mass detected (Zhuo et al., 2017). PLAC® mass assay has been shown mainly detecting surface LDL Lp-PLA_2 and Lp-PLA_2 associated with HDL which has smaller size than LDL (Zhuo et al., 2017).

In summary, our results demonstrate that rLp-PLA_2 tends to form association or oligomer with other hydrophobic substance in order to protect its substrate binding site from structure disruption. Our results explain why Lp-PLA_2 is found in association with lipoproteins in human circulation while the enzyme is mainly produced by hematopoietic and hepatic cells. The lipid particles such as LDL or HDL in human plasma possibly act as the chaperones to stabilize the Lp-PLA_2 in circulation by binding to its hydrophobic interface. Interaction of Lp-PLA_2 with Apo (a), ApoB100 and ApoA1 contributes significantly to the association of the enzyme with Lp(a), LDL or HDL. However, the phospholipid monolayer is the main driving force for the association of Lp-PLA_2 with lipoproteins. The protection of dilution inactivation of rLp-PLA_2 can be harnessed to study more details for the interaction of the protein with other lipoprotein components and specific phospholipids, such as negatively charged lipids. Such detail studies may reveal why Lp-PLA_2 selectively associates with LDL and HDL at the ratio about 2:1 (Gardner et al., 2008; Zhuo et al., 2017) and why it prefers to associate with negatively charged dense LDL. More researches are needed to answer these questions.

4. Experimental procedures

4.1. Materials

1-myristoyl-2-(4-nitrophenylsuccinyl)-sn-glycero-3-phosphocholine (14:0 NPSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The 10 × 300 mm Superose-6 column was manufactured by GE Healthcare Life Sciences (Piscataway, NJ). Rabbit anti-Lp-PLA_2 polyclonal antibodies were originally obtained from GlaxoSmithKline and also purchased from Cayman Chemicals (Ann Arbor, MI). Apolipoproteins were acquired from Biodesign (Saco, ME) or Lee BioSolutions (St. Louis, MO). Both recombinant and lipid-free human serum albumins (HSA) were obtained from Sigma-Aldrich (St. Louis, MO). Pig brain phospholipid, tri-glyceride, mdt-glyceride, cholesterol, cholesterol oleate and other chemicals were also purchased from Sigma-Aldrich. PLAC® mass and activity test kits for the quantitation of Lp-PLA_2 are the products of diaDexus Inc. Recombinant Lp-PLA_2 and C-terminal His-tag Lp-PLA_2 were also made by diaDexus Inc. as the components of PLAC® test kit. Other equipment or reagents were indicated in the text.

4.2. SDS-PAGE, western blotting and protein concentration determination

All SDS-PAGE were performed by using 4–12% Bis-Tris gradient gels (Invitrogen, San Diego). Gels were blotted on to nitrocellular membranes in a buffer (pH 7.5, containing 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA and 0.05 mM chlorobutanol) for 1 h at 50 V. Western blots were analyzed by using rabbit anti-Lp-PLA_2 polyclonal antibody or as indicated in the figures. All protein concentrations were determined by using either micro BCA or modified Bradford protein assays (Pierce Biotechnology) following the manufacturer's protocols. Both assays gave similar results for rLp-PLA_2.

4.3. HisGrap-Enzyme-Linked ImmunoSorbent Assay (HisGrap-ELISA) and PLAC® test assay

For HisGrap-ELISA, chromatography fractions were loaded and incubated in 96-well HisGrap nickel coated plates (Pierce Biotech, Rockford, IL) overnight with shaking. Plates were washed with 300 μl/well TBS, pH 7.4, containing 0.05% Tween-20 (TBS/T) for 6 times and incubated with 100 μl of primary rabbit polyclonal anti-Lp-PLA_2

antibody at 1 μg/ml each in the same TBS/T buffer containing 3% BSA and 0.1% Proclin-300 for 3 h at the room temperature. The plates were then washed as described before with the same TBS/T buffer and further incubated with 100 μl of the secondary antibody (goat anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA,) labeled with horseradish peroxidase (HRP) diluted at 1:15,000 in the same TBS/T/BSA buffer for 1 h. The plates were further washed 9 times with 300 μl/well of the same TBS/T buffer and incubated with 100 μl of TMB substrate for 5–20 minutes at the room temperature in dark. The reactions were stopped with 100 μl/well of 1 M HCl and concentrations were determined by reading of the plate in a SPECTRAmax M5 plate reader at 450 nm (Applied Biosystems, Foster City, CA).

For PLAC® mass test, briefly 1–40 μl (depending on the concentration) of each sample containing rLp-PLA_2 were applied onto the assay plate wells and the plate was incubated for 10 min at room temperature. Two hundred micro liters of the anti-rLp-PLA_2 antibody-HRP conjugate solution with or without 10 mM CHAPS were added to each well and the plate was incubated at room temperature for 3 h without sealing. The plate was then washed with TBS/T buffer for 4 times and incubated with 100 μl of TMB substrate solution for 20 min at the room temperature in dark. The reaction was stopped by adding 100 μl of 1 M HCl each well and concentrations were determined by reading of the plate in a SPECTRAmax M5 plate reader at 450 nm.

4.4. Enzyme kinetic assay and analysis

All of recombinant Lp-PLA_2 (rLp-PLA_2) enzyme kinetic assays in the study were carried out by using the PLAC® activity assay kit using 14:0 NPSPC as substrate developed by diaDexus, Inc. Basically, in a 96-well plate, reactions were started by adding 110–134 μl of the reaction buffer containing 0.54 mM 14:0 NPSPC to each well with 1–25 μl of Lp-PLA_2 samples according to the protocol by the manufacturer. The volumes of enzyme and reaction buffer were depended on the individual experiment. The reactions were followed at OD405 nm (absorbance) in a SPECTRAmax M5 plate reader and the steady state reaction rates of the first 3 or 5 min depending on the experiments were averaged. The data were processed and analyzed by using Microsoft Excel and GraphPad Prism.

4.5. Chemical cross-linking of rLp-PLA_2

Purified rLp-PLA_2 in 50 mM Tris, pH 8.0, with and without 10 mM CHAPS was diluted 1340 or 1460 fold to the final concentration of 1.0 μg/ml in 50 mM sodium phosphate with and without 10 mM CHAPS or 1% Tween-20, pH 7.6, containing 100 mM sodium chloride and 3 mM EGS. The mixtures were incubated at room temperature for 45 min and ethanol amine was added to the final concentration of 0.5 M to stop the reactions. The mixtures were then concentrated about 10 fold through a 20-kD cutoff iCON concentrator. Thirty μl of each sample were mixed with 10 μl of 4-fold SDS-PAGE loading buffer containing 200 mM DTT and 20 mM TCEP and incubated at 60 °C for 15 min and subjected to electrophoresis.

4.6. FPLC fractionation

Fractionation chromatography was carried out on an Akta10 or Akta100 by using a 10 mm x 300 mm Superose-6 column at room temperature with the flow rate of 0.3 ml per minute. Three different buffer systems (**A:** 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl, 2 mM EDTA and 0.01% sodium azide; **B:** PBS, pH 7.2; **C:** 50 mM Tris/HCl, pH 8.0) were used and no significant difference was observed. Fifty to two hundred μl of samples were injected per run depending on the Lp-PLA_2 concentrations of the samples after the column was equilibrated with the running buffer. Fraction collection was started at 21 min (the column void volume) after the sample injection and the collection volume was 0.6 ml/tube.

4.7. Preparation of LDL and HDL lipoproteins devoid of Lp-PLA_2 enzymatic activity

Concentrated human LDL and HDL were purchased from Lee BioSolutions in St. Louis. According to the manufacturer, LDL and HDL were prepared from fresh human plasma by undisclosed precipitation methods. Both the LDL and HDL showed one major band by Helena lipoprotein cellulose acetate electrophoresis. Characterization indicated that triglyceride/cholesterol ratios were 0.86 and 0.40 for LDL and HDL respectively. The lipoproteins were stored at -40 °C and shipped on dry ice. The purchased lipoproteins were thawed and subjected to inactivation of Lp-PLA_2 by incubation with 20 mM Pefabloc SC (Roche Applied Science, Indianapolis) in PBS, pH 7.2, at 4 °C overnight. The Pefabloc SC inactivated lipoproteins were then dialyzed extensively with a 10 kD cutoff membrane in 1000 fold volume excess of buffer containing 50 mM phosphate, pH 7.2, and 150 mM sodium chloride with 3 exchanges at 4 °C. The inactivated lipoproteins were found to have less than 10% of the original endogenous Lp-PLA_2 activity by the PLAC® activity assay. Both lipoproteins were further diluted to the desired concentrations before used in each experiment.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Contribution of authors

Roles of SZ include designing and carrying out experiments, drafting the manuscript and corresponding to submission. The works were completed during the time SZ working at diaDexus, Inc. Roles of CY include providing administrating suppor and funding, organizing and interprating data, drafting and editing manuscript draft.

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