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The Secretion and Uptake of Lysosomal Phospholipase A2 by Alveolar Macrophages

Akira Abe,* Robert Kelly,* Jessica Kollmeyer,* Miki Hiraoka,† Ye Lu,* and James A. Shayman2*†

Macrophages have long been known to secrete a Phospholipase A2 with an acidic pH optimum in response to phagocytic stimuli. However, the enzyme or enzymes responsible for this activity have not been identified. We report that mouse alveolar macrophages release lysosomal phospholipase A2 (LPLA2) into the medium of cultured cells following stimulation with zymosan. The release of the enzyme was detected by enzymatic activity assays as well as by Western blotting using an Ab against mouse LPLA2. LPLA2 is a high mannose type glycoprotein found in lysosomes, suggesting that the released enzyme might be reincorporated into alveolar macrophages via a mannose or mannose phosphate receptor. Recombinant glycosylated mouse LPLA2 produced by HEK293 cells was applied to LPLA2-deficient (LPLA2−/−) mouse alveolar macrophages. The uptake of exogenous LPLA2 into LPLA2−/− alveolar macrophages occurred in a concentration-dependent manner. The LPLA2 taken into the alveolar macrophages colocalized with the lysosomal marker, Lamp-1. This uptake was significantly suppressed in the presence of α-methylmannoside but not in the presence of mannose 6-phosphate. Thus, the predominant pathway for uptake of exogenous LPLA2 is via the mannose receptor, with subsequent translocation into acidic, Lamp-1-associated compartments. LPLA2−/− alveolar macrophages are characterized by marked accumulation of phosphatidylcholine and phosphatidylethanolamine. Treatment with the recombinant LPLA2 rescued the LPLA2−/− alveolar macrophages by markedly decreasing the phospholipid accumulation. The application of a catalytically inactive LPLA2 revealed that the enzymatic activity of LPLA2 was required for the phospholipid reduction. These studies identify LPLA2 as a high m.w.-secreted Phospholipase A2.

Lysosomes are intracellular organelles containing hydrolytic enzymes required for intracellular digestion of cellular and foreign materials such as cell fragments, microorganisms, surfactant, lipoproteins, and other pathogens. Over 40 distinct hydrolytic enzymes such as lipases, phospholipases, glycosidasases, proteases, nucleases, phosphatases, and sulfatasases are found in lysosomes. These enzymes are characterized by having acidic pH optima. In hematopoietic cells and melanocytes, the lysosomal compartment has dual functions that include degradation and secretion. Almost 30 years ago, macrophages were reported to possess an acid Phospholipase A2 that was secreted in response to phagocytic stimuli such as zymosan (1). This acidic phospholipase activity released from secretory lysosomes is distinct from that of the small secreted Phospholipase A2s (sPLA2s). These latter Phospholipase A2s are characterized by their low molecular weights (14–18 kDa) and presence of 5–8 disulfide bonds (2).

Unlike these sPLA2s, the biochemical characterization of the lysosomally secreted Phospholipase A2 is limited and its biological function unknown.

Our group previously reported the discovery of a novel enzyme, which has dual enzyme activities, short chain ceramide transacylation and Phospholipase A2, under acidic conditions (3). The preferred substrates for the enzyme are phosphatidylcholine and phosphatidylethanolamine. In the presence of a lipophilic alcohol such as ceramide as an acceptor for the fatty acyl group, the enzyme displays transacylase activity. In the presence of only water as an acceptor, the enzyme acts as a traditional Phospholipase A2. The enzyme was recovered in the soluble fraction obtained from cell homogenates and the lysosomal fraction of Madin-Darby canine kidney cells. Subsequently, we purified, cloned, and named the enzyme lysosomal Phospholipase A2 (LPLA2) (4, 5). Purified LPLA2 from bovine brain is a water-soluble glycoprotein consisting of a single polypeptide chain with a m.w. of 45 kDa. The protein exhibits acidic Ca2+-independent PLA and transacylase activities. LPLA2 is encoded by the same gene as lecithin-cholesterol acyl-transferase-like lysophospholipase (6). The primary structure of LPLA2 is highly preserved between mammalian and nonmammalian species. LPLA2 is post translationally modified both by a signal peptide cleavage and N-glycosylation. LPLA2 has 49% identity to lecithin-cholesterol acyl-transferase and belongs to the eν-hydrolase superfamily. The catalytic triad and four cysteine residues conserved in LPLA2 are preserved in lecithin-cholesterol acyl-transferase (7).

LPLA2 is ubiquitously expressed in all tissues studied to date, but is most highly expressed in terminally differentiated alveolar macrophages (AMs) (8). Recently, LPLA2−/− mice were generated by systemic deletion of exon 5 of the LPLA2 gene, which codes the catalytic site (9). These mice displayed a systemic lost of...
LPLA₂ activity. A marked accumulation of phospholipid was found in AM, peritoneal macrophages, and spleen in LPLA₂−/− mice at an early stage time. Electron micrographs revealed extensive lamellar inclusion bodies, a hallmark of cellular phospholipidosis, in the LPLA₂−/− mouse AM and PM. Therefore, LPLA₂ is thought to play an important role in cellular phospholipid homeostasis in those cells.

LPLA₂ activity has been found in the cell culture medium of LPLA₂ overexpressed cells, PMA-treated THP-1 cells, and 1-phenyl-2-decanoylamino-3-morpholino-propanol treated Madin-Darby canine kidney cells, and bronchoalveolar lavage cell-free fluid (Our unpublished data). These observations indicate that LPLA₂ is likely to be a secreted protein as well as a lysosomal protein, and suggests that the acidic Phospholipase A₂ activity released from macrophages when exposed to phagocytic stimuli derives from LPLA₂. In general, lysosomal enzymes contain oligosaccharide residues containing mannose and/or mannose 6-phosphate (10). Subsequently, secreted lysosomal enzymes are taken up by cells via a specific receptor mediated by carbohydrate recognition (11). Because LPLA₂ is a high mannose-type glycoprotein, the LPLA₂ released from the macrophages may be recovered by the cells via mannose or mannose 6-phosphate receptors.

Recently, we have generated purified recombinant mouse LPLA₂ and developed mAbs against mouse LPLA₂. In the present study, we explore whether LPLA₂ is released from mouse AMs following treatment with zymosan, and whether extracellular LPLA₂ is taken up to the AMs and trafficked back to the lysosome.

Materials and Methods

Reagents

2-Dioleoyl-sn-glycerol-3-phosphocholine was purchased from Avanti. N-acetylsphingosine (NAS) was obtained from Matreya. Endoglycosidase F1 was from Sigma-Aldrich. BCA protein assay reagent was from Pierce. HPTLC silica gel plates, 10 × 20 cm, were from Merck. Centriprep-10 (m.w. cut off 10,000) was from Amicon. The purified recombinant mouse LPLA₂ tagged with polyhistidine was obtained from Proteos. Anti-Lamp1, anti-early endosomal Ag 1 (EEA1), anti-Rab7, anti-calreulgin, and anti-translocase of the outer membrane of mitochondria (Tom20) rabbit polyclonal Abs were purchased from Santa Cruz Biotechnology. AlexaFluor 488 goat anti-rat IgG Ab, AlexaFluor 594 goat anti-rabbit IgG Ab, and Image-IT Signal Enhancer were obtained from Molecular Probes Invitrogen.

Preparation of cell homogenate from mouse alveolar macrophages

The cell homogenates of AMs were prepared as described (8). The adherent AMs (10⁵/well) on 6-well culture plates were washed three times with 2 ml of cold PBS and then treated under various conditions as described. The treated cells were washed three times with 2 ml cold PBS, scraped with a small volume of PBS, and transferred into a 15 ml glass tube. The cell suspension was disrupted by a probe type sonicator for 10 s for reduced fluorescence cross talk. The focal plane of the alveolar macrophage used for the images included was determined by first focusing on the DAPI blue staining of the nuclei. The depth of the image in the Z plane was 0.31 microns. For immunofluorescence staining, each condition was studied in triplicate, and three images were captured for each sample. The figures shown are representative.

Results

Localization of LPLA₂ in mouse AMs

Previously, we reported that in subcellular fractionation studies LPLA₂ localizes to lysosomes (5). To substantiate the results of the subcellular fractionation studies in an intact cell, immunofluorescence microscopic examinations were conducted using mouse AMs. AMs prepared from LPLA₂−/− mice and wild-type mice were treated with a mAb for LPLA₂ and stained with DAPI. The AMs from LPLA₂−/− mice showed no signal for LPLA₂, although...
FIGURE 1. Localization of LPLA2 in mouse AMs. A, AMs obtained from LPLA2−/− mice were fixed and incubated with a monoclonal anti-LPLA2 and polyclonal anti-Lamp 1 Abs. The cells were treated with Abs against LPLA2 (green) and stained with DAPI (blue). B, AMs from wild type mice were stained with DAPI and anti-LPLA2, as in A and with anti-Lamp-1 Ab (red). The merged image demonstrates colocalization of LPLA2 and Lamp-1. C, Merged images of wild type AMs stained for LPLA2 and the organelle markers EEA1, Rab7, calregulin, and Tom20.

The green LPLA2 and red Lamp-1 signals resulted in a yellow hue with the lysosomal marker Lamp-1 (red) (Fig. 1). These data suggest that the acidic Phospholipase A2 released from the port, the total activity of acidic Phospholipase A2 in the culture medium increased with time. The enzyme activity in the cell homogenate decreased in parallel with time. The total enzyme activity of LPLA2 in each well was constant. These results show that the reduction of the enzyme activity in the cell homogenate and the increase of the enzyme activity in the culture medium are dependent on the time of exposure to zymosan.

Immunoblotting using a mAb against mouse LPLA2 revealed that LPLA2 was detected in the cell homogenate and the cell cultured medium when the cells were treated for 1 h with 50 μg/ml zymosan (Fig. 2, E and F). The difference in molecular weights between the endogenous cellular LPLA2 and the LPLA2 standard reflects the presence of the presence of the polyhistidine tag on the recombinant LPLA2. The reduction in cell homogenate transacylase activity (Fig. 2F) is not apparent in the immunoblots (Fig. 2E) due to a difference in protein loading as described in the figure legend. The reduction of LPLA2 from mouse AMs treated with zymosan was confirmed with immunofluorescence microscopy. The LPLA2 signal (green) in the AMs decreased as the AMs ingested more zymosan in a time dependent manner (Fig. 2G). These immunofluorescence studies demonstrate an inverse relationship between LPLA2 signal and time of treatment with zymosan.

Release of LPLA2 activity from mouse AMs

As noted above, previous papers have reported that phagocytic stimuli induce AMs to secrete lysosomal acidic enzymes. The release of an acidic Phospholipase A2 activity was noted when the macrophages are treated with zymosan (1). According to this report, the total activity of acidic Phospholipase A2 in the culture system was constant. Additionally, our recent studies using LPLA2−/− mouse AMs demonstrated that >95% of the total activity of acidic Phospholipase A2 in the cell is due to LPLA2 (12). These data suggest that the acidic Phospholipase A2 released from AMs treated by zymosan could be LPLA2.

Mouse AMs were incubated with culture medium containing different concentrations of zymosan (Fig. 2, A and B). After 4-h incubation with zymosan, LPLA2 activity in the cell homogenate and cell culture medium was measured. The transacylase activity at pH 4.5 was used to determine the LPLA2 activity as described (14). Eighty-six percent of the total enzyme activity in the cell culture well was recovered in the cell homogenate when the AMs were treated with medium not containing zymosan. The enzyme activity was markedly reduced in the cell homogenate obtained from the cells treated with zymosan. The extent of the reduction was dependent on the concentration of zymosan. Approximately 40% of the total activity was recovered in AMs treated with 25 μg/ml zymosan. The total enzyme activity in each well was comparable (Fig. 2B). On the basis of these results, 50 μg/ml zymosan was chosen as the routine dose of incubation in subsequent experiments.

AMs were then treated with zymosan for different time periods (Fig. 2, C and D). The enzyme activity found in the cultured medium increased with time. The enzyme activity in the cell homogenate decreased in parallel with time. The total enzyme activity of LPLA2 in each well was constant. These results show that the reduction of the enzyme activity in the cell homogenate and the increase of the enzyme activity in the culture medium are dependent on the time of exposure to zymosan.

Incorporation and translocation of exogenous LPLA2 in LPLA2−/− mouse AMs

Many lysosomal enzymes are highly glycosylated proteins and are taken up by cells via a carbohydrate binding receptor. Previous studies showed that LPLA2 is bound to a Con A-conjugated resins, released from the resins with the buffer containing high concentration (500 mM) of mannose or α-methyl mannoside, and degraded by endoglycosidase F1 to a core peptide (4, 5). Additionally, depending on the species studied, three or four N-glycolation
sites are estimated to be present based on the primary structure of LPLA₂ (5). These findings indicate that LPLA₂ is a high mannose type glycoprotein. AMs express mannose and mannose 6-phosphate receptors on their plasma membrane. Thus, LPLA₂ released from AMs could potentially be incorporated into the cells via mannose or mannose 6-phosphate receptors.

**FIGURE 2.** Release of LPLA₂ from mouse AMs by treatment with zymosan. A–D and F, Transacylase activity. AMs isolated from wild-type mice were seeded in 6-well plate (10⁶ cells/well). The adherent cells on the plate were washed with PBS and treated with 0, 25, 50, and 75 µg/ml zymosan for 4 h at 37°C (A and B). C and D, The cells were treated with 50 µg/ml zymosan for 0.5, 1, and 2 h at 37°C. After the treatment, the cell homogenate (3.3 µg of protein) prepared from the treated AMs was incubated for 30 min at 37°C in citrate buffer (pH 4.5) with 40 µM NAS incorporated into phospholipid liposomes (PC/sulfatide/NAS (10:1:3.2 in molar ratio). The reaction product by the transacylase activity of LPLA₂, 1-O-acyl-NAS, was detected by TLC. The plate was developed in a solvent system consisting of chloroform/acetic acid (9:1, v/v) (A and C). E and F, The cells were treated with or without 50 µg/ml zymosan for 1 h. After the treatment, the transacylase activities in each cell homogenate (H) and medium (M) were determined as described above. E, Western blotting. Twenty and twenty-five micrograms of protein of cell homogenate obtained from the cells treated with 0 and 50 µg/ml zymosan, respectively, were separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane. For the medium, 50% of total volume was used for the electrophoresis. The membrane was incubated with a monoclonal anti-mouse LPLA₂ rat Ab. The Ag-Ab complexes on the membrane were visualized with an anti-rat IgG HRP-conjugated goat Ab using ECL reagents. The band intensity for the medium of the zymosan treated cells was weaker than that expected from the transacylase activity (F). However, this was due to absorption of LPLA₂ to the membrane of Centriprep that was used for reducing the volume of collected medium. G, The ingestion of zymosan particles and the location of LPLA₂ of the AMs. The cells were treated with 50 µg/ml zymosan for 1, 2, and 4 h at 37°C. After the treatment, the cells were fixed and incubated with a monoclonal anti-LPLA₂ Ab, treated Ab against LPLA₂ (green) and stained with DAPI (blue).
LPLA₂ into AMs. The recombinant mouse LPLA₂ used is glycosylated and has a poly-histidine C-terminal extension (13).

LPLA₂/H11002/H11002 mouse AMs were incubated for 1 day with culture medium containing various concentrations of the purified recombinant LPLA₂. The adherent AMs were washed extensively before analysis. A concentration dependent increase of the LPLA₂ enzyme activity recovered in the cell homogenate was observed (Fig. 3A, left panel). The LPLA₂ in the cell homogenate was measured by immunoblotting using a monoclonal anti-LPLA₂ Ab (Fig. 3A, right panel). An increase of the intensity of LPLA₂ protein bands detected by the mAb was accompanied with an increase of the exogenous recombinant enzyme (Fig. 3A, right panel), indicating that the recombinant LPLA₂ was effectively incorporated into cultured LPLA₂⁻/⁻ mouse AMs in a concentration dependent manner.

The predominant bands detected by a monoclonal anti-LPLA₂ Ab were smaller molecular size (45 kDa) than the intact recombinant LPLA₂ band (⁎ in Fig. 3A, right panel) and were not detected by a monoclonal anti-poly-histidine Ab (Fig. 3A, middle panel). The recombinant LPLA₂ bands tagged with a poly-histidine at the C terminus were detected in the samples obtained from 3 and 10 μg/ml recombinant LPLA₂-treated cells by the anti-LPLA₂ Ab although they are overlapped with the smaller ones (Fig. 3A, right panel). The recombinant LPLA₂ with a poly-histidine was detectable when the anti-polyhistidine Ab was used. However, the bands detected in the samples from 0.3 μg/ml and 1 μg/ml LPLA₂-treated cells were undetectable and very faint, respectively. The recombinant LPLA₂ of smaller molecular size was not detected in the cell culture medium in our assay system. These results demonstrate that the extracellular recombinant LPLA₂ is taken up by the cells and is cleaved at the C-terminal site in the cells. There was a good correlation between the profile of both the transacylase activity and the free fatty acid released in the homogenate (Fig. 3B) and that of the intensity of whole recombinant LPLA₂ (truncated plus nontruncated) (Fig. 3A, right panel). Thus the truncated LPLA₂ in the homogenate retains the LPLA₂ enzyme activity as measured by both ceramide acylation and free fatty acid release. On the basis of these results, 1 μg/ml recombinant LPLA₂ was chosen as the standard concentration of LPLA₂ added in subsequent experiments.

**FIGURE 3.** Incorporation of recombinant mouse LPLA₂ into mouse AMs. A. LPLA₂⁻/⁻ mouse AMs were treated with 0, 0.3, 1, 3, or 10 μg/ml recombinant mouse LPLA₂ for 1 day. After the treatment, the transacylase activity in cell homogenate (3.3 μg of protein) prepared from the treated AMs was determined as described in Fig. 2. Western blotting (Fig. 3A, right panel). Protein (70 μg) from each homogenate in A was separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with either monoclonal anti-poly-histidine Ab or a monoclonal anti-mouse LPLA₂ rat Ab. The Ag-Ab complexes on the membrane were visualized as described in Fig. 2. Purified recombinant LPLA₂ was applied to the far right lane and is denoted by an asterisk (3A middle and right-handed panels). B. LPLA₂ activity was measured as both the formation of 1-O-oleyl-N-acetylsphingosine and the release of free fatty acid. C. Incorporation of LPLA₂ incorporated into LPLA₂⁻/⁻ mouse AMs. LPLA₂⁻/⁻ mouse AMs were treated with or without 1 μg/ml recombinant mouse LPLA₂ for 1 day. After the treatment, the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-LPLA₂ and polyclonal anti-Lamp 1 Abs. The cells were then treated with Abs against LPLA₂ (green) and Lamp 1(red) and stained with DAPI (blue).
FIGURE 4. Effect of mannose derivatives on uptake of LPLA_2 into mouse AMs. A, Transacylase activity. LPLA_2−/− mouse AMs were preincubated in the presence or absence of 10 mM α-methyl mannose (Me-Man) or 10 mM mannose-6-phosphate (Man-6-P) for 30 min. LPLA_2−/− mouse AMs were treated with or without 1 μg/ml recombinant mouse LPLA_2 for 1 day. After the treatment, the transacylase activity in cell homogenate (4.0 μg of protein) prepared from the treated AMs was determined as described in Fig. 2. B, Western blotting. Protein (70 μg) from each homogenate in A was separated in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane. The membrane was incubated with a monoclonal anti-LPLA_2 Ab. Then the cells were fixed with paraformaldehyde and incubated with a monoclonal anti-MAb against LPLA_2 (green) and stained with DAPI (blue). The parallel panels reflect the results of two independent experiments.

To investigate cellular trafficking of the LPLA_2 taken into LPLA_2−/− mouse AMs, cells were incubated for 24 h with the recombinant LPLA_2 and stained with a mAb against LPLA_2 (Fig. 3B). A robust signal for LPLA_2 was detected with a staining pattern that was similar to the signal for endogenous LPLA_2 observed in wild-type AMs. The LPLA_2 incorporated into LPLA_2−/− mouse AMs colocalized with the lysosomal marker Lamp-1 producing a readily detectable yellow color caused by the overlapping green and red signals of the LPLA_2 and Lamp-1 respectively (Fig. 3, C and D).

Effect of mannose derivatives on uptake of exogenous LPLA_2 into LPLA_2−/− mouse AMs

To evaluate whether the mannose receptor or mannose 6-phosphate receptor is responsible for the LPLA_2 uptake by LPLA_2−/− mouse AMs, the LPLA_2−/− mouse AMs were treated with recombinant LPLA_2 in the presence of α-methyl mannose or mannose 6-phosphate. The enzymatic activity of LPLA_2 in the cell homogenate from LPLA_2−/− mouse AMs treated in the presence of 10 mM α-methyl mannose was significantly reduced, 69 ± 5% reduction compared with the control AMs (n = 3) (Fig. 4A). Additionally, the cell homogenate from LPLA_2−/− mouse AMs treated in the presence of mannose showed the comparable enzyme activity as the α-methyl mannose (data not shown). Western blot analysis showed a similar reduction in LPLA_2 when LPLA_2−/− mouse AMs were treated in the presence of α-methyl mannose (Fig. 4B). When LPLA_2−/− mouse AMs were treated in the presence of mannose 6-phosphate, no reduction was seen in enzymatic activity of LPLA_2, or the presence of LPLA_2, as shown in Fig. 4, A and B, respectively.

Additionally, immunofluorescence examinations of LPLA_2−/− mouse AMs in the presence of 10 mM α-methyl mannose or mannose 6-phosphate showed a parallel effect. Incorporation of the recombinant LPLA_2 by LPLA_2−/− mouse AMs was noticeably reduced in the presence of α-methyl mannose but not mannose 6-phosphate as shown in two independent experiments (Fig. 4C). These results indicate that either mannose or α-methyl mannose in the culture medium specifically inhibited the uptake of recombinant mouse LPLA_2 into LPLA_2−/− mouse AMs.

Reduction of phospholipid in LPLA_2−/− AMs by recombinant LPLA_2

The results above show that mouse AMs have secretion and re-uptake pathways of LPLA_2, and the LPLA_2 taken in the AMs could biologically and physiologically function. Hence, we considered that phospholipidosis of mouse AMs induced by a deficiency of LPLA_2 could be rescued by enzyme replacement treatment with the recombinant LPLA_2.

In 3-mo-old mice, the total phospholipid content of the LPLA_2−/− mouse AMs (593 nmol of phospholipid/mg of protein in Fig. 5A) was more than twice higher as compared with that of the wild-type mouse AMs (256 nmol of phospholipid/mg of protein in Fig. 5A). A marked accumulation of phospholipids, in particular phosphatidyethanolamine and phosphatidylcholine, except for sphingomyelin, was found in LPLA_2−/− mouse AMs (Fig. 5A). Phosphatidyethanolamine and phosphatidylcholine levels in the LPLA_2−/− mouse AMs were four and two times higher, respectively, than those of the wild-type mouse AMs. Electron micrographs in the LPLA_2−/− mouse AMs revealed the appearance of foam cells with lamellar inclusion bodies, a hallmark of cellular phospholipidosis (Fig. 5B).

LPLA_2−/− mouse AMs were treated with different concentrations of the recombinant mouse LPLA_2 for 1 day or 3 days (Fig. 5, C and D). Although the level of phospholipid in the AMs treated without the LPLA_2 decreased gradually during the treatment, the...
reduction of that in the AMs was markedly accelerated by treatment with the LPLA2 (Fig. 5C). After the treatment for 1 day, the phospholipid level of the LPLA2-treated cells was nearly reduced to that of wild type (Fig. 5, C and D). Interestingly, the phospholipid level in the LPLA2-treated cells declined substantially after one day of treatment, but little further change occurred with continued incubation for up to 3 days (Fig. 5C). Additionally, it was confirmed that the recombinant LPLA2 was incorporated into wild-type mouse AMs as observed in LPLA2−/− mouse AMs and that the level of phospholipid of the wild-type mouse AMs was almost unchanged by the LPLA2 treatment (data not shown). Furthermore, thin layer chromatography revealed that all accumulated phospholipids in LPLA2−/− mouse AMs were diminished by treatment with the recombinant LPLA2 (Fig. 5E). In all LPLA2-treated cases, the profile of phospholipids is quite similar to that of wild type (Fig. 5A).

The active catalytic site of LPLA2 is necessary for a decrease in accumulated phospholipids in LPLA2−/− mouse AMs

We sought to determine whether the enzymatic activity of LPLA2 incorporated is required to reduce the accumulated phospholipids in LPLA2−/− mouse AMs. The LPLA2 deficient cells were treated with recombinant mouse LPLA2 with or without a site directed mutation at the catalytic site. In the mutated LPLA2, the catalytic serine residue was converted to alanine (7). As reported previously, the wild-type mouse LPLA2 and the mutated mouse LPLA2 can be transiently expressed in transfected COS-7 cells. The soluble fraction prepared from the transfected cells was used for the treatment. Before initiating the enzyme treatment, immunoblotting was used to confirm that the expression level of LPLA2 protein found in the soluble fraction prepared from wild-type LPLA2 transfectants was very similar to that observed in the mutated LPLA2 transfectants (data not shown). LPLA2−/− mouse AMs were treated for 1 day with the same amount of protein of soluble fraction. A statistically significant decrease of phospholipid was measured in the AMs treated with the wild-type LPLA2 compared with vector alone (p = 0.0048) or to no treatment (p = 0.007, Fig. 6A).

FIGURE 5. Enzyme replacement treatment with recombinant LPLA2 for LPLA2 deficient mouse AMs. A, Phospholipid accumulation in AMs obtained from 3-mo-old wild-type and LPLA2−/− mice. The homogenates (30 μg of protein) prepared from AMs of 3-mo-old wild-type and LPLA2−/− mice were used for lipid extraction. Lipid extracts were applied to an HPTLC plate and separated in a solvent system consisting of chloroform/methanol/acetone/acetic acid/water (40/15/15/10/2, v/v). BMP, PG, PE, PS, PC, PI, and SM indicate bis(monoacylglycerol)-phosphate, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylycerine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin, respectively. B, Electron micrographs of AMs obtained from 3-mo-old wild-type and LPLA2−/− mice. C, LPLA2−/− mouse AMs were seeded into 6-well plates (10⁶ cells/well) and treated with or without recombinant mouse LPLA2 for 1 and 3 days. After the treatment, the cells were collected and cellular lipid extraction was conducted. D, LPLA2−/− mouse AMs seeded as described in C were treated with or without 1, 3, and 10 μg/ml recombinant mouse LPLA2 for 1 day. The homogenates (30 μg of protein) prepared from each treated-AMs were used for lipid extraction. E, Lipid extracts were applied to an HPTLC plate and separated in a solvent system consisting of chloroform/methanol/acetone/acetic acid/water (45/15/15/10/2, v/v). BMP, PG, PE, PS, PC, PI, and SM indicate bis(monoacylglycerol)-phosphate, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylycerine, phosphatidylcholine, phosphatidylinositol, and sphingomyelin, respectively.

FIGURE 6. Active catalytic site of LPLA2 is essential for reducing the accumulated phospholipid in LPLA2−/− mouse AMs. A, LPLA2-deficient mouse AMs were treated with or without the same amount of protein of the soluble fraction (16 μg protein/ml) obtained from COS-7 cells transfected with pcDNA3 (Vector), pcDNA3-tagged-mouse LPLA2 (LPLA2), or pcDNA3-tagged-mutated mouse LPLA2 (LPLA2 (mut)) for 1 day. In the LPLA2 (mut), the expression level of LPLA2 was similar to that of the mutated mouse LPLA2. After the treatment, the cells were collected and cellular lipids were extracted. B, Transacylase activity. The cell homogenate (12 μg of protein) prepared from the AMs treated in A was incubated for 60 min at 37°C in citrate buffer (pH 4.5) with 40 μM NAS incorporated into phospholipid liposomes (PC/sulfatide phosphate/NAS (10/1/3.2 in molar ratio).
The soluble fraction obtained from the mutated LPLA2 transfectants failed to decrease the accumulated phospholipid in the AMs (Fig. 6A). Additionally, a sizable enzymatic activity of LPLA2 was detected in the homogenate prepared from the AMs treated with the soluble fraction from wild-type LPLA2 transfectants (Fig. 6B).

**Discussion**

LPLA2 was originally identified by subtraction cloning in a model of foam cell formation in a macrophage cell line (6). The enzyme, originally termed LYPLA3, was characterized as a lysosphospholipid lipase. We independently identified the protein and gene as a ceramide acyltransferase with an acidic pH optimum (3, 5). In the absence of ceramide or lipophilic alcohol, the enzyme behaved as an acid Phospholipase A2. Because the Phospholipase A2 activity of the gene product is several orders of magnitude greater than the lysosphospholipid lipase, we have argued that the more appropriate appellation for the enzyme is lysosomal Phospholipase A2.

Among the Phospholipase A2, LPLA2 has been assigned as the first member of a new group among this superfamilly, group XV (16). LPLA2 is distinct from the fourteen other groups of Phospholipase A2 in having an acidic pH optimum and a lysosomal intracellular localization. However, the transacylase activity observed in LPLA2 is a characteristic of the group IV and VI Phospholipase A2 as well, although the acceptor specificity differs.

Several secreted Phospholipase A2s have been identified. These enzymes are primarily characterized by small molecular masses (13–19 kDa) and by the presence of five to eight disulfide bonds. These enzymes require millimolar levels of calcium for catalysis and use an active site histidine. A lone exception in terms of the molecular mass is the human and murine group III secreted Phospholipase A2 (17). This 55 kDa protein contains a group III domain. The enzyme is characterized by a pH optimum of 8, a substrate preference for phosphatidylglycerol, and calcium dependence.

In the present study, we report that LPLA2 is also a secreted high m.w. Phospholipase A2, but of lysosomal origin. Four previously published observations lend credence to this finding. First, an early study reported that peritoneal macrophages secrete a Phospholipase A2 activity in response to zymosan characterized by an acidic pH optimum (1). Second, recent studies on the mannose-6-phosphate plasma and urine glycproteomes reported that LPLA2 is recovered in both types of biological fluids (18, 19). Third, LPLA2 was identified in the Secreted Protein Discovery Initiative to be secreted (20). Fourth and finally, in the original paper describing the cloning of LYPLA3, protein was detected in human plasma in association with HDL lipoprotein (6). Based on these reports and the observation that 1-O-acylceramide transacylase activity was easily recovered in the cell medium of LPLA2-transfected cells, we sought to determine whether macrophages secreted LPLA2 in a regulated fashion.

We have previously observed that Phospholipase A2/transacylase activity can be measured in the medium of LPLA2-transfected COS-7 cells, THP-1 cells treated with PMA, and lung bronchoalveolar lavage cell-free fluid. The Phospholipase A2/transacylase activity that is detected under acidic conditions is specific for LPLA2 because LPLA2−/− mouse tissues, organs and macrophages lack this particular Phospholipase A2/transacylase activity (9). In addition, in mouse AMs >95% of the total acid Phospholipase A2 activity is attributable to LPLA2 (12). Therefore, we postulated that the acid Phospholipase A2 released from AMs by phagocytic stimuli is LPLA2.

In the present study we applied the ceramide transacylase activity assay, immunohistochemistry, and immunoblotting using a specific Ab against mouse LPLA2 to identify intracellular and extracellular LPLA2. These experiments confirmed that LPLA2 in mouse AMs localizes to endosomes as well as lysosomes and is secreted when stimulated with zymosan. The enzyme assay demonstrated that LPLA2 activity is lost from AMs treated with zymosan and is quantitatively recovered in the cultured medium. This indicates that LPLA2 is unlikely to be either transcriptionally or translationally regulated during the treatment with zymosan. Collectively, these data support the conclusion that the lysosomal acid Phospholipase A2 secreted from the zymosan-treated AMs is LPLA2.

The physiological role of LPLA2 released from the AMs is speculative. The creation and phenotyping of an LPLA2 null mouse by two groups provides some potential clues. The LPLA2 knockout mouse reported by our group was characterized by the onset within 3 mo of a foam cell phenotype in the alveolar macrophages and a modest accumulation of pulmonary surfactant (9). These findings are consistent with a role for LPLA2 in the catabolism of pulmonary surfactant, a substance containing high quantities of phosphatidylcholine and phosphatidylethanolamine, the primary substrates for LPLA2. Additionally, the lungs demonstrated a mononuclear cell associated perivascular and peribronchial inflammation. In mice older than 12 mo of age, splenomegaly and hepatomegaly was observed in association with the conversion of resident macrophages to foam cells within these organs. These data suggest that LPLA2 may have a role in long term immunoregulation. Taniyama and coworkers also described an LPLA2 knockout mouse (21). These mice, when bred on an apoE null background and fed a high fat diet, displayed an increase in the area of the aortic tree associated with atherosclerosis.

The mechanisms underlying these phenotypic changes are unknown. Although the knockout study supports a role for LPLA2 in the intracellular degradation of phospholipids, it remains possible that LPLA2 may have a role in the extracellular catabolism of phospholipids as well. Although the pH of most extracellular fluids is in the neutral range, extracellular pH may become acidic during ischemia or inflammation, such as with a consolidating pneumonia. Under normal physiological conditions, the extracellular pH may also decrease with the selective secretion of protons into a restricted pericellular compartment. Under such conditions, LPLA2 might degrade phosphatidylcholine or phosphatidylethanolamine forming the respective lyso-phospholipids and free fatty acid. Lyso-phosphatidylcholine, in particular, is recognized as an important ligand for G protein coupled receptors (22). Free fatty acids, notably arachidonic acid, may be biologically important as well. An older study on the activation of cytosolic and lysosomal Phospholipase A2 suggests that the latter may be important in zymosan-stimulated arachidonate and eicosanoid formation (23). With the identification of LPLA2 as the zymosan-stimulated, lysosome-associated Phospholipase A2, these findings should be reevaluated.

Most lysosomal enzymes are mannose-conjugated glycoproteins that are recovered via mannose and/or mannose 6-phosphate receptors on the cells. For example, α-galactosidase and acid sphingomyelinase are incorporated into cultured cells and organs via mannose 6-phosphate receptors on the plasma membrane (11). Because LPLA2 is a high mannose type glycoprotein, the binding and reincorporation of secreted LPLA2 by AMs via such carbohydrate binding receptors was not surprising.

In the present study, recombinant mouse LPLA2 was produced by HEK-293 cells and confirmed to be an N-glycoprotein by endoglycosidase F1 digestion (data not shown). This recombinant mouse LPLA2 was efficiently taken up to LPLA2−/− mouse AMs and significant fraction of the LPLA2 taken to the cells displayed limited degradation at the C-terminal region. The truncated LPLA2...
was associated with a m.w. of 45 kDa and retained enzymatic activity. Immunofluorescent staining revealed that the distribution of LPLA₂ incorporated into LPLA₂⁻/⁻ mouse AMs was similar to that of endogenous LPLA₂ in the wild-type AMs. Additionally, we observed that the LPLA₂ incorporated into the cells colocalized with Lamp-1, which localizes to late endosomes as well as lysosomes in AMs. The LPLA₂ not associated with Lamp-1 may also localize with early endosomes since the colocalization of LPLA₂ with an early endosome marker, EEA1, was observed in the wild-type AMs. These data indicate that the extracellular LPLA₂ is internalized via carbohydrate receptor mediated endocytotic pathway and targeted to acidic compartments including lysosomes and late endosomes.

As expected, the uptake of extracellular LPLA₂ by LPLA₂⁻/⁻ mouse AMs was significantly inhibited by mannose and α-methylmannoside but not mannose 6-phosphate. The 10 mM concentration used for the competition study was insufficient in completely blocking uptake, suggesting that affinity LPLA₂ and the mannose receptor is very high. Because AMs express mannose receptors, which recognize mannose-terminated glycoproteins, the predominant uptake pathway for extracellular LPLA₂ in AMs is likely via these receptors.

In lung, 20–30% of the pulmonary surfactant pool is taken up by AMs, sorted to lysosomes, and catabolized (24). Therefore, the defect of this catabolic pathway associated with loss of LPLA₂ activity results in phospholipidosis in those cells. Because LPLA₂⁻/⁻ mouse AMs demonstrate an early and robust foam cell phenotype, we explored whether recombinant LPLA₂ could rescue this phenotype in LPLA₂⁻/⁻ mouse AMs. LPLA₂⁻/⁻ mouse AMs treated with the recombinant mouse LPLA₂ showed a marked reduction of the accumulated cellular. Interestingly, the accumulated phospholipids in the LPLA₂⁻/⁻ mouse AMs gradually decreased with time even without any treatments. This observation possibly means that other phospholipases besides LPLA₂ are involved in catabolism for the accumulated phospholipids in the LPLA₂⁻/⁻ mouse AMs. Alternatively, AMs in culture might clear lysosomal phospholipids through exocytosis.

The phospholipid levels of the LPLA₂⁻/⁻ mouse AMs reached that of wild-type AMs by 24 h of exposure to recombinant LPLA₂. These levels did not change further after 24 h. A similar effect was observed when lower concentrations of the extracellular LPLA₂ (0.1–0.3 μg/ml in medium, data not shown) were used. These data suggest that the LPLA₂ taken up by the AMs degrades accumulated phospholipids, but does not result in the degradation of non-lysosomal associated phospholipid. Additionally, the rescue study using a catalytically inactive LPLA₂ demonstrated that enzymatically active LPLA₂ is essential for the reduction of the accumulated phospholipids. Thus, the extracellular LPLA₂ incorporated into AMs was translocated to the lysosome and retained its enzymatic activity.

The present study confirms that LPLA₂ in AMs is both a secreted enzyme as well as a lysosomal enzyme. LPLA₂ is released from AMs in response to zymosan. Additionally, once present in the extracellular compartment, LPLA₂ is incorporated into cells via a mannose receptor, internalized and translocated into acidic compartments, including endosomes and lysosomes, by a receptor-mediated endocytotic pathway. Finally, the enzyme rescue study indicates that the enzyme taken to the cell retains its catalytic function.

Disclosures

The authors have no financial conflict of interest.

References