Lysosomal Phospholipase A2 is Selectively Expressed in Alveolar Macrophages*

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Abstract

Lung surfactant is the surface-active agent comprised of phospholipids and proteins that lines pulmonary alveolae. Surfactant stabilizes the alveolar volume by reducing surface tension. Previously, we identified a phospholipase A2, termed LPLA2, with specificity towards phosphatidylcholine and phosphatidylethanolamine. The phospholipase is localized to lysosomes, is calcium independent, has an acidic pH optimum, and transacylates ceramide. Here, we demonstrate that LPLA2 is selectively expressed in alveolar macrophages but not in peritoneal macrophages, peripheral blood monocytes, or other tissues. Other macrophage-associated phospholipase A2s do not show a comparable distribution. LPLA2 is of high specific activity and recognizes disaturated-phosphatidylcholine as substrate. The lysosomal phospholipase A2 activity is six times lower in alveolar macrophages from mice with a targeted deletion of GM-CSF, a model of impaired surfactant catabolism, compared to those from wild type mice. However, LPLA2 activity and protein levels are expressed in GM-CSF null mice in which GM-CSF is expressed as a transgene under the control of the SP-C promoter. Thus LPLA2 may be a major enzyme of pulmonary surfactant phospholipid degradation by alveolar macrophages and may be deficient in disorders of surfactant metabolism.
Previously, in an attempt to identify the enzyme that transacylates ceramide at the 1-hydroxyl position, a novel phospholipase A2 was characterized (1). The phospholipase A2, termed 1-O-acylceramide synthase or lysosomal phospholipase A2 (LPLA2) has the following properties. In the presence of ceramide, the enzyme catalyzes the formation of 1-O-acylceramide by transacylation of fatty acids from the sn-2 position of phosphatidylcholine or phosphatidylethanolamine. In the absence of ceramide or other alcohols as acceptors, the enzyme acts as a traditional phospholipase A2. However, the phospholipase has a pH optimum of 4.5, is mannose rich, and is calcium independent (2). The phospholipase amino acid sequence is 49% identical to human LCAT (3). The homology with LCAT is highest within the catalytic domain, but the phospholipase does not recognize cholesterol as an acceptor for the fatty acid. The phospholipase colocalizes with other lysosomal proteins in cell fractionates. Upon the initial characterization of this enzyme, the functional role of this phospholipase A2 was not immediately apparent.

A role for an acidic phospholipase A2 activity has previously been suggested for the degradation of pulmonary surfactant phospholipids (4). The pulmonary acidic phospholipase A2 activity is also reported to be calcium independent and inhibited by a transition state analog of arachidonate, MJ33 (5). In rats treated with MJ33 the surfactant phospholipid catabolism was inhibited by approximately 40 to 50% suggesting that the drug sensitive phospholipase A2 activity contributes significantly to total surfactant degradation (6). In the present paper LPLA2 was studied in alveolar macrophages to determine whether this enzyme might play a role in pulmonary surfactant catabolism.
Materials and Methods

Reagents

Phosphatidylethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphorylcholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (DPPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Dicetyl phosphate and monoclonal anti-c-Myc clone 9E10 mouse ascites fluid were purchased from Sigma (St. Louis, MO); MJ33 was from Calbiochem (San Diego, CA); N-Acetyl-D-erythro-sphingosine (NAS) was from Matreya (Pleasant Gap, PA). BCA protein assay reagent was obtained from Pierce (Rockford, IL).

Isolation of rat cells and tissues

Respiratory disease-free female Wistar rats (125-150 g) were obtained from Charles River Laboratories, Inc. (Portage, MI) and housed under specific pathogen-free conditions. For isolation of alveolar macrophages by bronchoalveolar lavage, lavage buffer consisting of 0.15 M NaCl, 2.7 mM EDTA, 20 mM Hepes (pH 7.4), 5.5 mM dextrose, 1X antibiotic-antimycotic sol. (Invitrogen, Carlsbad, CA) was used. Following anesthesia with subcutaneous sodium pentobarbital, lungs were surgically excised and lavaged as reported (7). Peritoneal macrophages were obtained by lavage of peritoneal spaces with RPMI 1640 medium containing 1X antibiotic-antimycotic. Contaminated erythrocytes were removed by hypotonic lysis. Peripheral blood monocytes were isolated by centrifugation with Histopaque-1077 (Sigma, St. Louis, MO).

Cells were suspended in RPMI 1640 medium containing 1X antibiotic-antimycotic after counting number and plated in 100 mm culture dishes followed by incubation at 37°C in a humidified atmosphere of 5% CO2 in air. After 1 h, non-adherent
cells were removed by washing with phosphate buffered saline. Ninety-five percent of alveolar lavaged cells and 81% of peritoneal lavaged cells in the resultant adherent cell population were macrophages as confirmed by Wright-Giemsa staining (8). Greater than 90% of adherent peripheral blood mononuclear cells were monocytes (9).

Isolation of mouse cells and tissues

Wild-type C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). GM-CSF(−/−) was generated by Dranoff et al. (10). Bi-transgenic mice were generated from GM-CSF(−/−) mice by transgenic expression of a chimeric gene containing GM-CSF under the surfactant protein C (SP-C) promoter (SP-C-GM mice) (11). The specificity of the SP-C promoter results in targeted expression of GM-CSF by type II alveolar epithelial cells. Founder GM-CSF(−/−) and SP-C-GM mice were kindly provided by Dr. J. Whitsett (Children’s Hospital, Cincinnati, OH). After anesthesia with intraperitoneal sodium pentobarbital, the trachea was cannulated and the lung was lavaged with phosphate buffered saline containing 0.5 mM EDTA as previously described (12). The lavage fluid of each group was pooled, and the cell pellet was collected by centrifugation. All mice were housed in specific pathogen-free conditions. Mice were used at 3-5 months of age. All experiments were approved by the University of Michigan Committee on the Use and Care of Animals.

RNA extraction and cDNA synthesis

Total RNA was extracted from each rat organ using TRIzol reagent (Invitrogen) followed by purification using RNeasy kit (Qiagen, Valencia, CA). For isolated cells, total RNA was extracted using RNeasy kit. Total RNA was used to synthesize cDNA with oligo(dT)12-18 primers and in the Super Script First Stand synthesis system (Invitrogen).
**Primers and standard plasmid for real time PCR**

Primers were designed from the LPLA2 (GenBank accession #AY490816), Prdx6 (#NM053576), and iPLA2 (XM346803) gene sequences respectively. The rat iPLA2 gene sequence was deduced from its amino acid sequence (#P97570). The primer sets were as follows: LPLA2 forward (5’-ACATGCTCTACTTTCATGCGG-3’) and reverse (5’-AGAAGCACACGTTTCAGATA-3’), Prdx6 forward (5’-CAGTGTGCACCACAGAACTTG-3’) and reverse (5’-AGCTCTTTGGTGAAGACTCCT-3’), iPLA2 forward (5’-ACTACATCTGGCCTGCCGCAA-3’) and reverse (5’-AGAAGCATTCCGCGCCATCTC-3’).

Standard plasmids were generated with the respective PCR products of LPLA2, Prdx6, and iPLA2 ligated into the pCR4-TOPO vector (Invitrogen) and followed by cloning, purification, quantification, and sequencing.

**Quantitative analysis of the LPLA2, Prdx 6, and iPLA2 mRNA expression by real time PCR**

A standard curve for each primer set was generated by a serial dilution of a TOPO vector containing each partial gene sequence. One µl of synthesized cDNA mixture was used for a real-time PCR. The Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN) containing SYBR Green (Molecular Probes, Eugene, OR) was used for PCR reaction mixture. The PCR amplifications employed 40 cycles with steps at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. The iCycler instrument (BioRad Laboratories, Hercules, CA) was used to perform PCR and analyze data. The quality and
quantity of each RNA were determined by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). The mRNA concentrations were normalized to the total RNA.

**Preparation of the soluble fraction from rat alveolar and peritoneal macrophages, peripheral blood mononuclear cells and tissues.**

In preparation of the soluble fractions of alveolar macrophages, peritoneal macrophages, and peripheral blood monocytes, the adherent cells on the culture dishes were washed three times with 8 ml of cold phosphate buffered saline, scraped with a small volume of phosphate buffered saline and transferred into a 15 ml plastic tube. The cells were collected by centrifuge at 800g for 10 min at 4°C, re-suspended with 0.4-1.0 ml of cold 0.25 M sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA and disrupted by a probe type sonicator for 10 sec x 5 at 0°C. The suspension was centrifuged for 1 h at 100,000g at 4°C. The resultant supernatant was passed through a 0.2 µm filter and used as a soluble fraction.

For the preparation of rat tissue soluble fractions, each tissue was washed with cold phosphate buffered saline, weighed and homogenized by a Potter Elvehjem-type homogenizer with cold 0.25 M sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA to obtain 10% homogenate. The homogenate was centrifuged for 10 min at 600g at 4°C. The resultant supernatant was sonicated by a probe type sonicator for 10 sec x 5 at 0°C and centrifuged for 1 h at 100,000g at 4°C. The supernatant was passed through a 0.2 µm filter and used as a soluble fraction.

For the preparation of the soluble fraction of mouse alveolar macrophages, the macrophages were collected from wild-type (C57BL/6), GM-CSF (-/-), and SP-C-GM mice by whole lung lavage and were pooled as describe above. The cell pellets were
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washed 3 times with cold phosphate buffered saline and resuspended in cold 0.25 M sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA. The suspension was disrupted by a probe type sonicator and followed the same procedure as described above.

**Enzyme assay (transacylase activity).**

Phospholipids, DOPC and PE, and N-acetylsphingosine (NAS) were used in the assay system as a donor and an acceptor, respectively, of an acyl group. The transacylase activity was determined by analysis of 1-O-acyl-N-acetylsphingosine formation rate. The reaction mixture consisted of 45 mM sodium citrate (pH 4.5), 10 µg/ml bovine serum albumin, 40 µM NAS incorporated into phospholipid liposomes (DOPC/PE/dicetyl phosphate/NAS (5:2:1:2 in molar ratio)) and soluble fraction (0.7-10 µg) in a total volume of 500 µl. The reaction was initiated by adding the soluble fraction, kept for 5-60 min at 37°C and terminated by adding 3 ml of chloroform/methanol (2:1) plus 0.3 ml of 0.9 %(w/v) NaCl. The mixture was centrifuged for 5 min at room temperature. The resultant lower layer was transferred into another glass tube and dried down under a stream of nitrogen gas. The dried lipid dissolved in 40 µl of chloroform/methanol (2:1) was applied on an HPTLC plate and developed in a solvent system consisting of chloroform/acetic acid (9:1). The platted was dried down and soaked in 8 %%(w/v) CuSO₄, 5H₂O, 6.8 %(v/v) H₃PO₄, 32 %(v/v) methanol. The uniformly wet plate was briefly dried down by a hair dryer and charred for 15 min in a 150°C oven. The plate was scanned and the amount of the reaction products was estimated by NIH-image 1.62.

**Dipalmitoylphosphatidylcholine degradation studies**

The reaction mixture consisted of 47 mM sodium citrate (pH 4.5), 10 µg/ml bovine serum albumin, liposomes (130 µM phospholipids) and 2.59 µg/ml of the soluble
fraction of rat alveolar macrophages in a total volume of 500 µl. The liposomes consisted of dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, and dicetylphosphate in a molar ratio of 50:50:16. Trace 1-palmitoyl-2-[1-\textsuperscript{14}C]palmitoyl-L-3-phosphatidylcholine (2.55 x 10\textsuperscript{5} cpm/assay) was added. The reaction was initiated by the addition of 15 µl of the soluble macrophage fraction or sucrose buffer and incubated for 40, 80, and 120 min at 37°C. The reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1) plus 300 µl of 0.9% NaCl. The resultant lower layer was transferred into a small glass tube and dried down under a stream of nitrogen gas. The dried lipid was redissolved with chloroform/methanol (2:1) and applied to high performance thin layer chromatography plates. The free fatty acid and lyso-phosphatidylcholine were separated in solvent systems consisting of chloroform/methanol/acetic acid (98:2:1) and chloroform/methanol/water (60:35:8) respectively. Each plate was dried and sprayed with 10 µg/ml primulin in acetone/water (60:40). The products were detected under ultraviolet light, scraped, and transferred into scintillation vials. The silica gel was dispersed in 5 ml of ECOLUME (ICN Biomedicals) by a sonic bath and counted.

**Immunoblotting**

The soluble fraction was precipitated by the method of Bensadoun and Weinstein (13). The resultant pellet was dissolved with 30 µl of loading buffer consisting of 125 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 2% 2-mercaptoethanol, and 10 µg/ml bromophenol blue in addition to 1.5 µl of 2 M Tris for SDS polyacrylamide gel electrophoresis. Proteins were separated using a 12% acrylamide gel and transferred to a PVDF membrane using the transfer buffer (20 mM Tris, 150 mM glycine in 20% methanol) at constant voltage 100 volts for 3 h at 4°C. The membrane was incubated
with an anti-mouse LPLA2 peptide ($^{100}$RTSRATQFPD) rabbit serum and monoclonal anti-c-Myc mouse ascites fluid. The antigen-antibody complex on the membrane was visualized with an anti-rabbit IgG HRP- conjugated goat antibody or an anti-mouse IgG HRP-conjugated goat antibody using diaminobenzidene and hydrogen peroxide.
Results

A series of tissues from the Wistar rat were isolated and assayed for lysosomal phospholipase A2 activity (Fig. 1A). Because the lysosomal phospholipase A2 can transacylate ceramide at the 1-hydroxyl position, the transacylase activity was determined as the formation of 1-O-acyl-N-acetylsphingosine (1-O-acyl-NAS). The enzyme activity was comparable in a wide range of tissues including brain, kidney, spleen, thymus, and lung. Of the tissues assayed, the specific activity was highest in thymus and spleen, suggestive that hematopoietic cells might be a source of higher enzyme activity.

Pulmonary alveolar macrophages were next studied. When pulmonary alveolar macrophages were isolated by bronchoalveolar lavage and assayed for phospholipase A2 activity, a greater than 40-fold higher activity of the lipase was observed compared to other tissues. This difference was not present in either peritoneal macrophages or peripheral blood monocytes.

A comparison of the enzyme activity between peritoneal macrophages, peripheral blood monocytes, and alveolar macrophages was made. The higher LPLA2 activity in alveolar macrophages compared to peritoneal macrophages and monocytes was evidenced by the formation of 1-O-acyl-N-acetylsphingosine and free fatty acid (Fig. 1B). Because the initial velocity of the reaction in the alveolar macrophage was significantly greater than that observed in other cells and tissues, the reaction time was shortened to 5 minutes from 30 minutes and the assay protein lowered to 0.9 µg from 10 µg (Fig. 1C). Under these conditions, the phospholipase A2 activity was linear. The enzyme activities in the peritoneal macrophages and monocytes were slightly higher but comparable to those observed in the other tissues and significantly less than that
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measured in the alveolar macrophages. These data suggest that elevated lysosomal phospholipase A2 is a marker of the terminally differentiated alveolar macrophage.

We also evaluated the mRNA expression of LPLA2 in the tissues and macrophages using real time PCR (Fig. 1D). A good correspondence was observed in the mRNA levels normalized to total RNA and the transacylase activity. A comparison was also made between LPLA2 and another reported acidic phospholipase A2. This phospholipase, termed aiPLA2, was identified as the same protein as 1-cys-peroxiredoxin (Prdx6), a non-selenium glutathione peroxidase (14,15). The mRNA levels of Prdx6 were not significantly greater in the alveolar macrophage compared to other tissues. Another macrophage associated phospholipase A2 is the calcium independent group VIA enzyme termed iPLA2 (16). mRNA levels of iPLA2 were also not significantly greater in the alveolar macrophage compared to other tissues.

We next sought to demonstrate that the high transacylase/phospholipase A2 activity present in the alveolar macrophage was in fact LPLA2. A polyclonal antibody was raised to a peptide corresponding to the sequence $^{100}$RTSRATQFPD of the mouse LPLA2 protein. An immunoblot of the soluble protein fractions of rat peritoneal and alveolar macrophages was compared to that of c-Myc-tagged mouse LPLA2 expressed in COS-7 cells (Fig 2A). The immunoblot identified a major band in the alveolar macrophage protein fraction of the predicted molecular weight. No corresponding band was detected in the peritoneal macrophage fraction or when preimmune serum was used. The antibody recognized the c-Myc-tagged protein as well. The identity of the c-Myc-LPLA2 was confirmed with an anti-c-Myc antibody. Densitometric measurements of the detected bands demonstrated a ratio of 1:0.57 between the alveolar macrophage LPLA2
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and the mouse LPLA2 (Fig. 2B). A comparison of reaction velocities was also made between the endogenous enzyme and the expressed LPLA2 (Fig. 2C). The ratio of the reaction velocities was 1:0.58. These data suggest that the transacylase/phospholipase A2 activity measured in the alveolar macrophage was due to LPLA2.

The transacylase and phospholipase A2 activities in the alveolar macrophage were further evaluated for their calcium dependence and pH optima (Fig. 3A). No phospholipase A2 or transacylase activities were observed at pH 7.4 in the presence or absence of calcium. The absence of activity persisted even when the reaction was followed for up to 60 minutes. At pH 4.5 both the formation of 1-O-acyl-N-acetylsphingosine and free fatty acid were observed.

The majority of pulmonary surfactant phospholipid is in the form of dipalmitoylphosphatidylcholine. Thus it should be demonstrable that this disaturated lipid is a suitable substrate for LPLA2. Dipalmitoylphosphatidylcholine has a phase transition temperature of 41°C. Therefore, liposomes containing dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and dicetyl phosphate were used (Fig. 3B). Under these conditions and in the absence of N-acetylsphingosine as an acceptor, the release of both palmitic acid and oleic acid was observed (Fig. 3C). In order to ascertain the relative contributions of phospholipase A1 and A2 activities in the degradation of the disaturated phosphatidylcholine, a liposomal mixture containing sn-2 labeled phosphatidylcholine was incubated with a soluble fraction obtained from the alveolar macrophages (Fig. 3D). In the absence of the soluble fraction, the spontaneous but significant release of radiolabeled palmitic acid and lyso-phosphatidylcholine occurred in a time dependent manner (42 cpm/min and 26 cpm/min for palmitic acid and lyso-phosphatidylcholine
respectively). In the presence of the alveolar macrophage fraction, significant amounts of radioactive palmitic acid and lyso-phosphatidylcholine were produced. Because the dipalmitoylphosphatidylcholine was labeled at the C2 position, the radiolabeled lyso-phosphatidylchoine detected represented \( \text{sn}-2 \)-lyso-phosphatidylcholine, the product of phospholipase A1 activity. Under these conditions, the rate of formation of \( \text{sn}-2 \)-lyso-phosphatidylcholine was two times that of palmitic acid formation. Therefore 33 and 66 percent of the palmitic acid released from dipalmitoylphosphatidylcholine in this assay system is due to the respective activities of phospholipase A2 and phospholipase A1.

In our original characterization of LPLA2 we noted that the enzyme was insensitive to the phospholipase A2 inhibitors bromoenol lactone and nonadecyltetraenyl trifluoromethyl ketone. However, MJ33, an inhibitor demonstrated to block surfactant phosphatidylcholine catabolism \textit{in vivo}, was not evaluated. The LPLA2 activity was measured in the presence of this compound. A concentration dependent inhibition of the transacylase activity was observed (Fig. 4). A comparable response was noted for the expressed c-Myc-tagged mouse LPLA2.

Pulmonary alveolar proteinosis is a disorder of impaired catabolism of surfactant phospholipids and proteins. The GM-CSF null mouse was discovered to exhibit a phenotype consistent with this human disease. These mice display excess surfactant accumulation in the lungs associated with the engorgement of lipids within alveolar macrophages (10). This phenotype is reversed in bi-transgenic mice that are GM-CSF null but express GM-CSF under the control of the SP-C promoter (11). LPLA2 activity was measured in the alveolar macrophages of C57BL/6 mice, GM-CSF null mice, and bi-transgenic mice that express GM-CSF under the control of the surfactant protein C
promoter (Fig. 5A). LPLA2 activity, as measured by the time dependent increase in 1-O-
acyl-N-acetylsphingosine, was readily apparent in the wild-type mouse macrophages. However, this activity was six times lower in macrophages from the null mice. The GM-
CSF bi-transgenic mouse macrophages demonstrated somewhat higher LPLA2 activity. Interestingly, cholesterol levels were very high in the GM-CSF-/- mouse macrophages but not detected in the wild type of transgenic mice. An immunoblot of the proteins obtained from the isolated alveolar macrophages of the respective mice displayed parallel changes (Fig. 5B). No LPLA2 protein was detected in the GM-CSF -/- mice in our assay system. LPLA2 levels in the bi-transgenic macrophages were slightly higher than in the wild-type mice.

We next attempted to determine whether GM-CSF alone was sufficient to result in LPLA2 expression in the alveolar macrophages from the null mice. GM-CSF-/-
alveolar macrophages were isolated and cultured for up to 5 days in the presence or absence of GM-CSF (10 ng/ml). No change in LPLA2 activity was observed in the treated macrophages even when the reaction was followed for 60 minutes (Fig. 6).
Discussion

There are three significant findings in the present study. First, LPLA2, an acidic phospholipase of unknown function, is highly expressed in alveolar macrophages. While LPLA2 expression and activity is ubiquitously found in all tissues assayed, the mRNA levels and enzyme activity is robustly present in the pulmonary alveolar macrophage. The LPLA2 activity in peripheral blood monocytes and peritoneal macrophages is comparably low. These data are consistent with the interpretation that LPLA2 is a marker of the differentiated alveolar macrophage.

Second, LPLA2 recognizes disaturated phosphatidylcholines as substrates. Dipalmitoylphosphatidylcholine is the major component of pulmonary surfactant. Phosphatidylcholine and phosphatidylethanolamine respectively account for 84.1% and 1.9% of the surfactant lipid in the C57BL/6 mouse (17). Therefore, LPLA2 may potentially serve as the primary catabolic enzyme of surfactant phospholipids.

Third, LPLA2 activity is very low in alveolar macrophages from GM-CSF-/- mice. These mice have been characterized as a model of pulmonary alveolar proteinosis, a disorder of impaired surfactant catabolism. The activity is restored in GM-CSF-/- mice in which GM-CSF is expressed in type II alveolar epithelial cells under the control of the SP-C promoter. Thus LPLA2 may be a link between GM-CSF deficiency and impaired surfactant lipid degradation.

The enzyme or enzymes responsible for the catabolism of surfactant phospholipids has been the object of prior studies. These reports have suggested that degradation of dipalmitoylphosphatidylcholine occurs either through the activity of an undefined calcium independent phospholipase A1 or through the activity of a calcium
independent phospholipase A2 that has optimal activity at an acidic pH (18). Our data indicate that both activities are present in the alveolar macrophage.

The existence of an acidic phospholipase A2 activity was first described over 25 years ago (19,20). Such an activity was subsequently described in rabbit lung and in rat and human alveolar macrophages. Fisher and colleagues identified such an activity in lung secretory lamellar bodies and in lysosomes (21). They further reported that this activity was inhibited by the phospholipid transition state analogue, MJ33 (6). In support of this observation they demonstrated that MJ33 inhibited DPPC degradation by 50% in isolated perfused rat lungs. Using MJ33 they isolated a protein of molecular weight 15 kDa (21) and subsequently at 26 kDa (14) as the putative lysosomal phospholipase A2.

There are several reasons why aiPLA2 is unlikely to be the enzyme accounting for acidic phospholipase A2 activity that catalyzes the degradation of surfactant phospholipids. aiPLA2 has low specific activity; aiPLA2 is a cytosolic enzyme; aiPLA2 lacks the mannose groups typically seen in lysosomally targeted proteins; the tissue distribution of aiPLA2 does not favor the lung, monocytes, or macrophages; for aiPLA2 to function as a phospholipase it would have to be a dual function enzyme with two separate catalytic domains, one for the lipase and one for glutathione peroxidase; and gene targeting of aiPLA2 does not result in a pulmonary phenotype consistent with aberrant surfactant metabolism (22,23).

The acidic lysosomal transacylase is a novel type of phospholipase A2 that is structurally homologous to LCAT (3). The chromosomal location of the lysosomal phospholipase A2 gene on 16q22 close to LCAT suggests that the lipase arose as a gene duplication product of LCAT. Because the lysosomal phospholipase A2 lacks activity
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toward cholesterol as an acceptor for the sn-2 fatty acids of phosphatidylcholine and phosphatidylethanolamine, the functional significance of this enzyme was not immediately apparent at the time it was first identified. The markedly increased expression and activity of the phospholipase A2 observed in alveolar macrophages suggest that the primary function of this enzyme is likely to be the degradation of glycerophospholipids present in pulmonary surfactant. Besides the absence of LPLA2 activity in GM-CSF-/- mice, two additional observations support this view.

First, the specific activity of the phospholipase A2 is considerably higher than that reported for other candidate enzymes. These include Prdx6/aiPLA2 and the calcium independent neutral macrophage associate PLA2. Second, the enzyme is inhibited by MJ33, an inhibitor previously demonstrated to block the majority of dipalmitoylphosphatidylcholine degradation in murine lung.

Finally, deletion of the gene for GM-CSF or its receptor in mice results in pathological accumulation surfactant in the lungs. This accumulation is not a consequence of increased production of surfactant components, but appears to be linked to impaired surfactant catabolism by alveolar macrophages (24). Our observation that LPLA2 is activity is expressed at high level in alveolar macrophages from wild-type mice but very low in alveolar macrophages from GM-CSF null mice with impaired surfactant clearance further supports the hypothesis that LPLA2 plays a critical role in surfactant phospholipid catabolism. Short term treatment of GM-CSF null alveolar macrophages in vitro with GM-CSF was not sufficient to induce LPLA2 expression, although transgenic expression of GM-CSF exclusively in the lungs of GM-CSF null mice did result in high level alveolar macrophage expression of LPLA2. This finding is not surprising, insofar
as long-term pharmacologic treatment with nebulized GM-CSF or gene transfer has been required to reverse the pathologic finding of pulmonary alveolar proteinosis in GM-CSF deficient mice. Clearly GM-CSF is necessary but not sufficient to induce alveolar macrophage differentiation.

An additional unexplained finding was the presence of high cholesterol levels in the alveolar macrophages of the GM-CSF null mice. This observation does not appear to have been previously reported in the phenotypic characterization of these mice. While the mechanism for this accumulation is not apparent, an association between GM-CSF and cholesterol metabolism has been previously reported (25).

This observation is also consistent with a recently reported study in which our group evaluated the role of agonists that induce macrophage differentiation on LPLA2 expression in THP-1 cells (26). In this study we observed that phorbol ester and all trans retinoic acid induced LPLA2 transcription, but that GM-CSF was without effect. Sorting out those signals that regulate both alveolar macrophage differentiation and LPLA2 expression will require additional work.

Thus we have demonstrated the robust expression of an acidic lysosomal phospholipase A2 within the alveolar macrophage, the primary site of surfactant degradation. The low expression and activity of this phospholipase A2 in a model of pulmonary alveolar proteinosis raises the possibility that this phospholipase may mediate human disorders associated with abnormal surfactant metabolism.

1. Abbreviations: NAS, \( N \)-Acetyl-D-erythro-sphingosine; GM-CSF, granulocyte macrophage colony stimulating factor; LCAT, lecithin cholesterol acyltransferase;
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LPLA2, lysosomal phospholipase A2; MJ33, 1-hexadecyl-3-trifluoroethylglycerol-\textit{sn}-2-phosphomethanol.

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References


Figure legends

**Fig. 1.** The distribution LPLA2 activity and mRNA in rat tissues and macrophages.

(A) The distribution of LPLA2 transacylase activity in rat tissues and macrophages. The soluble fraction obtained from each tissue (20 µg of protein/ml) and alveolar macrophages (AM), peritoneal macrophages (PM), or peripheral blood monocytes (PBM) (2 µg of protein/ml) were assayed in citrate buffer, pH 4.5, with 40 µM N-acetyl-sphingosine (NAS) and the enzyme activity was measured as the formation of 1-O-acyl-NAS as described in the Methods section. The data represent the mean ± S.D. of three independent determinations. For lung, the tissue was processed after the macrophages were obtained by bronchoalveolar lavage.

(B) Transacylase and phospholipase A2 activities in the soluble fraction obtained from rat alveolar macrophages (AM), peritoneal macrophages (PM) and peripheral blood monocytes (PBM). The soluble cell fractions obtained from AM (1.8 µg protein/ml), PM (6.0 µg protein/ml), and PBM (18 µg protein/ml) were assayed in citrate buffer, pH 4.5, with 40 µM N-acetyl-sphingosine (NAS). The formation of 1-O-acyl-NAS and free fatty acid was determined following extraction and separation by thin layer chromatography as described in the Methods section. Panel (C) represents the time dependent formation of 1-O-acyl-N-acetylsphingosine in rat macrophages and monocytes.

(D) The mRNA levels of lysosomal phospholipase A2 (LPLA2), peroxiredoxin 6 (Prdx6), and cytosolic calcium-independent phospholipase A2 (iPLA2) in rat tissues. Real time PCR was performed to measure the mRNA of LPLA2, peroxiredoxin 6 (Prdx6), and iPLA2 in each tissue or cell type as described in the Methods section.
Fig. 2. Expression of LPLA2 protein in rat macrophages.

(A) Immunoblot analysis of the soluble fraction of AM and PM. The soluble cell fraction (20 µg of total protein) obtained from AM, PM, and c-Myc-tagged mouse LPLA2 over-expressed COS7 cells were separated by SDS polyacrylamide gel electrophoresis and subjected to immunoblotting with a rabbit polyclonal antibody raised to mouse LPLA2 peptide (\textsuperscript{100}RTSRATQFPD) and a monoclonal antibody to c-Myc. LPLA2 and c-Myc-tagged LPLA2 were detected as described in the Methods section. mLPLA2 denotes c-Myc-tagged mouse LPLA2 expressed in COS7 cells. (B) The densitometric profile of the AM and mLPLA2 lanes from panel A. The ratio of AM band area to mLPLA2 band area was 1: 0.57. (C) The transacylase activity of the soluble fraction of AM compared with that of mLPLA2. The ratio of the initial velocity of 1-\textit{O}-acyl-NAS formation in the AM soluble fraction to that of mLPLA2 soluble fraction was 1: 0.58.

Fig. 3. Characterization of alveolar macrophage LPLA2 activity.

(A) Effect of pH and calcium on the transacylase activity in rat AM. The soluble fraction (1.50 µg of protein/ml) of rat AM was assayed in 47 mM sodium citrate (pH 4.5) or in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) with 40 µM NAS at 37°C for the indicated time periods. Under the neutral conditions, the reaction mixture containing 1 mM EDTA or 1 mM CaCl\textsubscript{2} was used for the assay. The reaction products were separated by thin layer chromatography as described in the Methods section.

(B and C) Degradation of dipalmitoylphosphatidylcholine by rat alveolar macrophages. The soluble fraction (3.14 µg of protein/mg) of rat AM was incubated in citrate buffer, pH 4.5, with 130 µM phospholipid in liposomes consisting of DOPC: DPPC: dicetyl...
phosphate (the molar ratio of 3.07: 3.07: 1) at 37°C for indicated time period. The released free fatty acids in the reaction were separated by a silver nitrate impregnated HPTLC plate that was developed a solvent system consisting of chloroform/acetic acid (95:5). In panel C the released free fatty acid by the soluble fraction was corrected by subtracting the fatty acid released in the absence of the soluble fraction at each time point and plotted against incubation time. (D) Metabolism of 1-palmitoyl-2-[1-14C]palmitoyl-L-3-phosphatidylcholine by rat alveolar macrophages. The liposomes consisted of dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, and dicetylphosphate in a molar ratio of 50:50:16. with the addition of trace 1-palmitoyl-2-[1-14C]palmitoyl-L-3-phosphatidylcholine (2.55 x 10^5 cpm/assay). The reaction was initiated by the addition of 15 µl of the soluble macrophage fraction or sucrose buffer and incubated for 40, 80, and 120 min at 37°C. The products were extracted, separated, and quantified as detailed in the Methods section.

**Fig. 4.** The effect of MJ33 on LPLA2 transacylation activity in rat alveolar macrophages and c-Myc-tagged mouse LPLA2 transfected COS 7 cells. The soluble fraction (2.42 µg of protein/ml) prepared from rat AM and that (2.26 µg of protein/ml) from the LPLA2 transfected cells were assayed in citrate buffer, pH 4.5, with 40 µM NAS in liposomal form with different concentrations of MJ33. The enzyme activity was determined as described in the Methods section. The enzyme activity in the absence of MJ33 was used as the control.
Fig. 5. The expression of LPLA2 transacylase activity and protein in GM-CSF (-/-) and SP-C-GM mice macrophages. (A) Protein from the soluble fractions obtained from C57BL/6, GM-CSF (-/-) and SP-C-GM mouse macrophages were assayed for the transacylation of NAS. The specific activities of LPLA2 from C57BL/6, GM-CSF (-/-) and SP-C-GM mouse macrophages were 9.50 ± 0.055, 1.58 ± 0.081 and 19.4 ± 1.40 µg/min/mg of protein, respectively. (B) Immunoblot analysis of the soluble fraction of each soluble fraction. The soluble fraction proteins (18.7 µg) were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting. For the mLPLA2 lane, the soluble fraction was prepared from c-Myc-tagged mouse LPLA2 expressed in COS7 cells. The LPLA2 densitometric ratios of the C57BL/6 band to that of the SP-C-GM band was 1: 1.96.

Fig. 6. GM-CSF has no *in vitro* effect on LPLA2 activity in alveolar macrophages from GM-CSF-/- mice. Non-adherent alveolar macrophages (ca. 4 X 10^6 cells/well) obtained from GM-CSF-/- mice were seeded onto 6-well plates containing 2 ml of DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cells were treated with or without 10 ng/ml GM-CSF for 1, 3, and 5 days at 37°C in a CO2 incubator. The treated cells were collected into a 15 ml plastic tube and washed 3 times with 2 ml of cold phosphate buffered saline. The cellular soluble fraction was prepared and the transacylase activity measured as described in the Methods section. Four mg of the soluble fraction was incubated with liposomes containing NAS for 30 and 60 min at 37°C under acidic conditions.
Fig. 1
Fig. 2

A

B

C

FORMATION OF 1-O-ACYL-NAS (µg/µg protein)

INCUBATION TIME (min)

0.0 0.2 0.4 0.6 0.8

0 5 10 15 20

AM

mLPLA2

Kda

Std PM AM mLPLA2

Anti-LPLA2 Anti-c-Myc

100 71 44 29

Std

mLPLA2

mLPLA2

AM

mLPLA2
Fig. 3
Fig. 5