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Lysosomal Phospholipase A2 and Phospholipidosis

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A lysosomal phospholipase A2, LPLA2, was recently characterized and shown to have substrate specificity for phosphatidylcholine and phosphatidylethanolamine. LPLA2 is ubiquitously expressed but is most highly expressed in alveolar macrophages. Double conditional gene targeting was employed to elucidate the function of LPLA2. LPLA2-deficient mice (Lpla2−/−) were generated by the systemic deletion of exon 5 of the Lpla2 gene, which encodes the lipase motif essential for the LPLA2 activity. The survival of the Lpla2−/− mice was normal. Lpla2−/− mouse mating pairs yielded normal litter sizes, indicating that the gene deficiency did not impair fertility or fecundity. Alveolar macrophages from wild-type but not Lpla2−/− mice readily degraded radiolabeled phosphatidylcholine. A marked accumulation of phospholipids, in particular phosphatidylethanolamine and phosphatidylcholine, was found in the alveolar macrophages, the peritoneal macrophages, and the spleens of Lpla2−/− mice. By 1 year of age, Lpla2−/− mice demonstrated marked splenomegaly and increased lung surfactant phospholipid levels. Ultrastructural examination of Lpla2−/− mice showed alveolar and peritoneal macrophages revealed the appearance of foam cells with lamellar inclusion bodies, a hallmark of cellular phospholipidosis. Thus, a deficiency of lysosomal phospholipase A2 results in foam cell formation, surfactant lipid accumulation, splenomegaly, and phospholipidosis in mice.

Materials and Methods

Reagents. Synthetic phospholipids, including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (POPC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:1-14:1 PC); 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine (15:0-15:0 PE); 1,2-dipenta-decanoyl-sn-glycero-3-phosphoglycerol (15:0-15:0 PG); 1,2-dimyristoyl-sn-glycero-3-phosphoserine (14:0-14:0 PS); 1-heptadecanoyl-2-hydroxyl-sn-glycero-3-phosphocholine (17:0 lysoPC); and N-heptadecanoyl ceramide (N17:1 Cer) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Deuterated palmitic acid (d16:0 FA) was purchased from Cambrex Isotope Laboratories (Andover, MA). Dicetyl phosphate was purchased from Sigma (St. Louis, MO). 1-palmitoyl-2-[3H]oleoyl-sn-glycero-3-phosphocholine (25 μCi/ml) was from Amersham Biosciences (Piscataway, NJ), and N-acetyl-l-erythro-sphingosine (NAS) was from Matreya (Pleasant Gap, PA). Bicinchoninic acid protein assay reagent was obtained from Pierce Chemical (Rockford, IL).

Generation of Lpla2 double conditional targeted mice. The genome sequence containing the Lpla2 gene was obtained from screening the Resgen C37 embryonic stem (ES) cell line BAC clone library (Invitrogen, Carlsbad, CA). A Smal-SacI fragment of approximately 8,450 bp of the Lpla2 gene was subcloned into the pUC vector. In a previous report it was shown that the lipase motif, located within exon 5, is essential for LPLA2 enzyme activity (12). Therefore, to create the conditional null allele, the SpeI-DraI region containing exon 5 was flanked with two loxp sites and then inserted into the vector (Fig. 1A). The PGK-neo cassette flanked with two FRT sites was inserted at the SpeI site in the intron between exons 4 and 5 in reverse orientation. The targeting vector was sequenced to ensure that no mutation had been introduced and then was linearized by NdeI digestion and electroporated into C37 ES cells. Homologous recombinant clones were obtained from G418-resistant colonies screened at a frequency of 20%. The G418-resistant clones were screened by PCR using primers inside and outside the targeting construct. A correctly targeted clone was injected into C57BL/6 blastocysts. The chimera mice were mated with C57BL/6 to obtain heterozygous mice carrying the targeted allele. Mice carrying the targeted allele were mated with C5BL/6 to obtain heterozygous mice carrying the targeted allele. Mice carrying the targeted allele were mated with Cre mice of C57BL/6 background (stock no. 3724; The Jackson Laboratory) to delete the neo cassette. The conditional heterozygous mice were then mated with EIIa Cre mice of C57BL/6 background (stock no. 3724; The Jackson Laboratory) to excise the region containing exon 5. The heterozygous mice carrying the null allele were mated to generate homozygous (+/−), heterozygous (+/+), and wild-type (+/+ ) littermates of the Lpla2 null allele. Homologous recombination at the null allele was screened by PCR (Fig. 1B). Genomic DNA was extracted from the tails of the mice. The PCR primers were as follows: a, 5′-CAGGGTA GTCACAAGCTCGGATG-3′; b, 5′-CAAAAGCTCTGGACTGTTT-3′; c, 6139

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allele was generated by Cre-mediated excision. The heterozygous mice carrying conditional alleles were mated with EIIa Cre liver; S, spleen; K, kidney; T, thymus; B, brain; Lu, lung. D. Immunoblots of Lpla2 from the alveolar macrophages obtained from Lpla2 PCR was performed using primers which are able to cover the Lpla2 coding region. M, molecular marker. M indicates molecular marker. C. Reverse transcription-PCR assay. Total RNAs were isolated from various mouse organs and synthesized cDNA. recombination. The primers a and d as well as b and c were used for upper panel and lower panel assays, respectively. TV indicates targeting vector. delete exon 5. B. Genotype analysis by PCR. Genomic DNA was extracted from mouse tail, and PCR was performed to evaluate homologous recombination. The primers used for PCR are shown as horizontal arrows with letters. The conditional allele was generated by Flp-mediated excision. The heterozygous mice carrying targeted alleles were mated with Ella Cre transgenic mice to delete exon 5. B. Genotype analysis by PCR. Genomic DNA was extracted from mouse tail, and PCR was performed to evaluate homologous recombination. The primers a and d as well as b and c were used for upper panel and lower panel assays, respectively. TV indicates targeting vector. M indicates molecular marker. C. Reverse transcription-PCR assay. Total RNAs were isolated from various mouse organs and synthesized cDNA, PCR was performed using primers which are able to cover the Lpla2 coding region. M, molecular marker d_174 RF DNA/HaeIII; H, heart; Li, liver; S, spleen; K, kidney; T, thymus; B, brain; Lu, lung. D. Immunoblots of Lpla2 from the alveolar macrophages obtained from Lpla2+/−, Lpla2+/-, and Lpla2−/− mice. Alveolar macrophages were isolated from mice as described in Materials and Methods, and the soluble fraction was separated by gel electrophoresis as previously described (1). A rabbit anti-LPLA2 polyclonal antibody (100RTSRATQFPD109) was used for detection. E. Transacylase activity in the soluble fraction of alveolar macrophages obtained from Lpla2+/−, Lpla2+/−, and Lpla2−/− mice. Each soluble fraction (3 μg of protein) obtained from 3-month-old Lpla2+/-, Lpla2+/-, and Lpla2−/− mouse alveolar macrophages was incubated for 30 min at 37°C in citrate buffer, pH 4.5, with 40 μM NAS in liposomal form, and formation of 1-O-acyl-NAS was determined as described in Materials and Methods. The transacylase specific activities for the Lpla2+/−, Lpla2+/-, and Lpla2−/− mouse alveolar macrophages were 3.9 μg/min/mg protein, 2.08 μg/min/mg protein, and undetected, respectively. w/o, without; Fr., fraction.

Reverse transcription-PCR analysis. Total RNA was isolated from each mouse organ using Trizol reagent (Invitrogen) followed by purification using an RNeasy kit (QIAGEN, Valencia, CA). The total RNA was used to synthesize cDNA with oligo(dT)12-18 primers in the SuperScript First-Strand synthesis system (Invitrogen). Primers used for PCR were 5'-ATGGATGCGCCATCTC-3' (forward) and 5'-TCAAGGTTCAGAGCACACGTITT-3' (reverse). PCR was performed using ExTaq polymerase with the conditions described above. PCR products were purified and sequenced.

Isolation of mouse macrophages and tissues. All mice were housed in specific-pathogen-free conditions and used at 2 to 12 months of age. After anesthesia with CO2 inhalation, the organs were isolated. For isolation of alveolar macrophages, the trachea were cannulated and the lungs were lavaged with phos-
Lipid analysis. Lipids were extracted from tissues, alveolar macrophages, peritoneal macrophages, and bronchoalveolar lavage fluid of 3-month-old mice by the partially modified method of Bligh and Dyer (5). The phospholipid content was measured by the method of Ames (4). Individual phospholipids were separated by high-performance thin-layer chromatography and quantified as described above. Disaturated phosphatidylcholine in samples was isolated by the method of Mason et al. (13). Whole-lipid extract (20 to 100 nmol as phospho-
lipid) was dried under a stream of nitrogen gas. The dried lipid was dissolved in 500 µl of 0.2 mol/l sodium tetraborate in carbon tetrachloride and incubated for 15 min at room temperature. The reaction mixture was dried under a stream of nitrogen gas, resuspended in 500 µl of chloroform/methanol (20:1, vol/vol), and applied to an aluminia column (0.6 g of activated neutral aluminum oxide, 150 mesh) preequilibrated with chloroform/methanol (20:1, vol/vol). The column was washed with 8 ml of chloroform/methanol (20:1, vol/vol). Disaturated phosphatidylcholine was eluted with 4 ml of chloroform/methanol/7% ammonium hydroxide (70:30:2, vol/vol), and the eluted fraction was dried under a stream of nitrogen gas. Phosphatidic acid was eluted by the method of Ames (4).

For electrospray ionization/mass spectrometry (ESI/MS), lipid extraction from alveolar macrophage preparations (0.1 to 0.5 mg of proteins) was performed using a modified method of Bligh and Dyer as previously described in detail (11). Briefly, internal standards, including 14:0-14:0 phosphatidylserine (8.0 nmol/mg protein), 15:0-15:0 phosphatidylglycerol (6 nmol/mg protein), 15:0-15:0 PE (13 nmol/mg protein), 14:0-14:0 PA (14 nmol/mg protein), 17:0 lysoPC (2 nmol/mg protein), N17:1 ceramide (0.24 nmol/mg protein), and d18:0 fatty acid (12 nmol/mg protein), were added to each individual sample based on protein concentration. Lipids from each sample were extracted against 2 ml of 50% MeOH twice, back extracted against 2 ml of 10 mM LiCl twice, filtered with a 0.2-µm polycarbonate membrane filter, and finally stored in chloroform/methanol (1:1, vol/vol) at a concentration of 600 µg/ml. The lipid extracts were finally flushed with nitrogen, capped, and stored at −20°C for ESI/MS analyses (typically within 1 week). Each lipid solution was further diluted approximately 10-fold just prior to infusion and lipid analysis. To this diluted solution, LIQH (50 nmol/mg of protein) was added just prior to performing further lipid analyses in both negative- and positive-ion modes.

ESI/MS analyses were performed utilizing a triple-quadrupole mass spectrometer (ThermoElectron TSQ Quantum Ultra, San Jose, CA) equipped with an electrospray ion source as described previously (11). Typically, a 1-min period of signal averaging in the positive mode was employed for each MS spectrum, and a 1- to 2-min period of signal averaging for each MS/MS spectrum was used. Global analyses of lipid extracts were performed by shotgun lipidomics as described previously (9, 10). Quantitation of each individual molecular species of lipid classes was performed using a two-step process, as described in detail previously (8, 9).

Degradation of 1-palmitoyl-2-14C[14C]oleoyl-glycero-3-phosphocholine (14C-PC) by alveolar macrophages. Alveolar macrophages (1.3 × 106 cells) obtained from 3- to 5-month-old Lpl2+/− mice and Lpl2−/− mice were seeded into a 35-mm dish containing 2 ml of RPMI 1640 medium (Invitrogen) containing 1% antibiotic-antimycotic and followed by incubation at 37°C in a humidified atmosphere of 5% CO2 in air. After 90 min, nonadherent cells were removed by washing with PBS. The adherent cells were incubated with 2.1 ml of RPMI 1640 containing 20 mM medium containing 320 µM (0.25 µCi/ml) 14C-labeled POPE in liposomes consisting of POPC/diCer (10:1 molar ratio) for 4 h at 37°C. After the incubation, the cells were washed three times with 2 ml of cold PBS and fixed with 1 ml of cold methanol. The fixed cells were scanned and transferred into a glass tube. An additional 1 ml of methanol was used to recover the remaining cells in the dish. The cell suspension was mixed with 1 ml chloroform plus 0.8 ml of 0.9% NaCl and sonicated in a water bath sonicator briefly and kept for 1 h at room temperature. The mixture was centrifuged for 30 min at 2,000 × g at room temperature, and the supernatant was transferred into a long glass tube. The supernatant was washed with water and centrifuged for 5 min at 800 × g. The lower layer was washed with 2 ml of methanol plus 1.6 ml of 0.9% NaCl, centrifuged for 5 min at 800 × g, and washed again with 2 ml of methanol plus 1.6 ml of water. The resultant lower layer was transferred into another glass tube and dried under a stream of nitrogen gas. The dried lipid was dissolved in 100 µl of chloroform/methanol (2:1, vol/vol). Half of the lipid extract was applied to an HPTLC plate and developed in a solvent system consisting of chloroform/acetic acid (9:1, vol/vol) or chloroform/methanol/water (60:35:8, vol/vol). After development, the plate was dried, sprayed with ENHANCE, and exposed on X-ray film at −80°C for 4 days.

Nucleotide sequence accession number. The genome sequence containing the Lpl2a gene has been submitted to GenBank under accession number AY179884.
RESULTS

Generation of LPLA2-deficient mice. To create Lpla2 null mice, a targeting vector was designed and constructed containing two loxP sites and two FRT sites with a PGK-neo cassette placed between the FRT sites for modification by use of Cre/loxP and Flp/FRT recombination systems (Fig. 1A). Exon 5, which encodes the lipase motif essential for Lpla2 activity, was floxed with two loxP sites and then inserted into the vector. CJ7 ES cells were electroporated with the linearized targeting vector. Homologous recombinant clones were obtained from G418-resistant colonies screened at a frequency of 20%. A correctly targeted clone was injected into C57BL/6 blastocysts. The chimeric mice were mated with C57BL/6 mice to obtain heterozygous mice carrying the targeted allele.

Mice carrying the targeted allele were found to be normal and fertile. However, homozygous offspring from heterozygous pairs showed a modest reduction of Lpla2 activity in the soluble fraction of brain (data not shown). This finding suggested that the neo cassette inclusion affected Lpla2 expression. flp1 transgenic mice express Flp recombinase in the early embryo under the control of the human β-actin promoter. Mice with the targeted allele were mated with flp1 transgenic mice to delete the neo cassette. The allele in which the neo cassette was deleted by using an Flp/FRT recombination system is termed the “conditional allele.” Mice carrying the conditional allele were normal in appearance and were fertile. The Lpla2 enzyme activity in the homozygous mice carrying the conditional allele was found to be the same as that of wild-type mice (data not shown). EIIa Cre transgenic mice express Cre recombinase in the one-cell zygote stage of embryo development under the control of the adenovirus EIIa promoter. Homozygous mice carrying the conditional allele were mated with EIIa Cre transgenic mice to excise the region containing exon 5. The resultant heterozygous mice carrying the null allele were mated together to generate Lpla2+/−, Lpla2−/−, and Lpla2+/+ litters. Homologous recombination at the null allele was screened by PCR (Fig. 1B). The predicted product from the deletion of the loxP site flanking region was detected in both Lpla2−/− and Lpla2+/+ but not in the Lpla2+/+ mice.

Lpla2+/− mice were viable and fertile. They produced an average of 8.7 pups per litter with a normal Mendelian frequency, indicating no selective fetal or neonatal loss of homozygous pups. Survival of the Lpla2−/− mice was normal. Lpla2−/− mating pairs gave normal litter sizes (8.5 pups per litter), indicating that the gene deficiency did not grossly impair fertility or fecundity. Screening of Lpla2 mRNA expression in seven organs from Lpla2−/− mice demonstrated the deletion of exon 5 in each organ (Fig. 1C), indicating that the deletion was systemic.

An immunoblot was performed on the protein extracts from alveolar macrophages of Lpla2−/−, Lpla2+/−, and Lpla2+/+ mice using a previously generated rabbit polyclonal antibody raised to mouse Lpla2 peptide (19RSRATQFPD) (1). A single band at a molecular mass of ca. 44 kDa was observed in blots from Lpla2+/+ and Lpla2−/− macrophages. However, no protein was detected from Lpla2−/− alveolar macrophages (Fig. 1D). The Lpla2 enzyme activities were also compared among genotypes. The transacylase activity, as measured by the formation of 1-O-acetyl-N-acetylphosphoglycerine, was absent in Lpla2−/− mouse alveolar macrophages (Fig. 1E). The transacylase activity from Lpla2+/+ mouse alveolar macrophages was approximately 50% of that of the Lpla2+/+ mouse alveolar macrophages. The deficiency of the enzyme activity in the soluble fraction of the Lpla2−/− mouse was also observed in other cells and tissues, including peritoneal macrophages, heart, lung, liver, spleen, kidney, thymus, and brain (data not shown).

Phospholipid degradation in alveolar macrophages. Many classes of phospholipase A2 exist (17). On the one hand, the absence of ceramide transacylase activity in alveolar macrophages might not necessarily mean that cellular phospholipase A2 activity would be impaired as well. On the other hand, Lpla2 is very highly expressed in alveolar macrophages and might represent the major phospholipase A2 activity. Therefore, the degradation of PC was more extensively evaluated in the mouse alveolar macrophages. A choice of substrate was required. When previously studied, Lpla2 was observed to recognize 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (DPPC), a major component of pulmonary surfactant lipid, when presented as a substrate in DOPC/DPPC liposomes (1). However, Lpla2 demonstrated greater activity toward DOPC than DPPC. Furthermore, DPPC led to a reduction of the enzyme activity on DOPC in DOPC/DPPC liposomes. These results suggested that unsaturated phospholipids are better substrates than saturated phospholipids and may provide a preferable environment in the Lpla2 reaction.

The transfer of oleic acid to N-acetyl-sphingosine and the release of oleic acid from POPC by POPC by the soluble fraction obtained from Lpla2−/− mouse alveolar macrophages were observed when POPC/dicetyl phosphate/N-acetyl-sphingosine liposomes were used (Fig. 2A). The transacylase activity in the POPC liposome system was comparable to that observed with the DOPC/dicetyl phosphate/N-acetyl-sphingosine liposome system. These results were further evaluated with the use of alveolar macrophages and radiolabeled POPC. Using 1-palmitoyl-2-14C oleoyl-sn-glycero-3-phosphorylcholine, the phospholipase A2 activity was compared between the lungs and alveolar macrophages of Lpla2+/+ and Lpla2−/− mice (Fig. 2B). When assayed at pH 4.5, the phospholipase A2 activity in the wild-type lung was 1.10 nmol/min/mg protein but was only 0.072 nmol/min/mg protein in the Lpla2 null mice. The alveolar macrophage activity was 18.6 nmol/min/mg protein versus 0.43 nmol/min/mg protein in the wild-type and knockout mice, respectively. The higher specific activity of Lpla2 in the alveolar macrophages compared to that of the whole lung is consistent with the prior observation reported for Lpla2 assayed as ceramide transacylation (1).

The products of the phospholipase A2 reaction were further evaluated. The radioactive oleic acid released from POPC was readily detected in the lipid extract obtained from the Lpla2+/+ mouse alveolar macrophages treated with 1-palmitoyl-2-[14C] oleoyl-sn-glycero-3-phosphorylcholine/dicetyl phosphate liposomes (Fig. 2C). On the contrary, there was no radioactive oleic acid detected in the lipid extract obtained from the Lpla2−/− mouse alveolar macrophages treated with 14C-labeled POPC liposomes. The total radioactivity found in the lipid extract, obtained from the Lpla2−/− mouse alveolar macrophages, was approximately one-half of the amount from the Lpla2+/+ mouse macrophages. The radioactivity of oleic acid...
recovered from the TLC plate was 260 cpm and 50 cpm for Lpla2\(+/-\) and Lpla2\(-/-\) mouse macrophages, respectively (Fig. 2C). Additionally, the released radioactive oleic acid was comparably low (40 cpm on the TLC plate) when the \(^{14}\text{C}\)-labeled POPC liposomes were incubated with the cultured medium without alveolar macrophages. Thus, 10 times less radioactive oleic acid was released from the Lpla2\(-/-\) mouse alveolar macrophages compared to that released from the Lpla2\(+/-\) mouse macrophages. Therefore, most of the oleic acid released from POPC in the Lpla2\(+/-\) mouse alveolar macrophages was due to Lpla2 activity and not an alternative phospholipase A2.

Interestingly, the radioactive lysoPC was detected in Lpla2\(+/-\) and Lpla2\(-/-\) alveolar macrophages. This metabolite, labeled in the sn-2 position, is produced by phospholipase A1. The radioactivity of lysoPC was 150 cpm and 100 cpm, respectively, for Lpla2\(+/-\) and Lpla2\(-/-\) mouse alveolar macrophages. These results indicate that the degradation of phospholipid in Lpla2\(-/-\) mouse alveolar macrophages is most greatly impaired due to a lack of phospholipase A2 activity.

**Phospholipid accumulation in the Lpla2\(-/-\) mouse.** The phospholipid content and profile in alveolar and peritoneal macrophages and other tissues of 3-month-old Lpla2\(+/-\) and Lpla2\(-/-\) mice were next examined. The total phospholipid content of the Lpla2\(-/-\) mouse alveolar macrophages was more than two times higher than that of the Lpla2\(+/-\) mouse alveolar macrophages. Thin-layer chromatography of the lipid extract of the alveolar macrophages showed a marked accumulation of both PE and PC in the Lpla2\(-/-\) mouse alveolar macrophages. The phospholipid in the Lpla2\(-/-\) mouse alveolar macrophages was most greatly impaired due to a lack of phospholipase A2 activity.
(305 nmol of phospholipid/mg of protein) was 40% higher than that of the Lpla2+/+ peritoneal macrophages (223 nmol of phospholipid/mg of protein). A similar change in phospholipid profile was observed in the Lpla2−/− peritoneal macrophages.

ESI/MS analysis was used to quantify the lipid content of the Lpla2+/+ and Lpla2−/− alveolar macrophages. Alveolar macrophages were isolated from 12-month-old mice, and internal standards consisting of 14:0-14:0 phosphatidylserine, 15:0-15:0 phosphatidylglycerol, 15:0-15:0 phosphatidylethanolamine, 14:1-14:1 phosphatidylcholine, 17:0 lysophosphatidylcholine, N17:1 ceramide, and d16:0 fatty acid were added to each individual sample based on protein concentration. Marked increases in PC and PE were detected in the null mouse macrophages (Table 1; also see the supplemental material). By contrast, no increase in sphingomyelin or phosphatic acid was measured. Lyosphatidylcholine content increased in the null mouse macrophages. However, the total lyosphatidylcholine concentration was less than 1% of that of phosphatidylycholine. In wild-type mouse macrophages, 65% of the phosphatidylcholine had one or more fatty acids consisting of the palmitic species of phosphatidylcholine by Lpla2.

**DISCUSSION**

We successfully generated Lpla2−/− mice using a double conditional targeting system. Lpla2−/− mice generated by the systemic deletion of exon 5 of the Lpla2 gene, which encodes the lipase motif essential for lysosomal phospholipase A2 activity, were healthy at birth and fertile. Lpla2−/− mice showed no systemic lysosomal phospholipase A2 activity and a characteristic accumulation of PE and PC in alveolar macrophages, peritoneal macrophages, and spleen. A similar trend in the phospholipid profile was also observed in tissues such as liver and lung but with less accumulation. The selective accumulation of PE and PC in Lpla2−/− mice is consistent with the previously reported substrate specificity of Lpla2. Both phospholipids are preferred substrates of Lpla2 (2). Electron microscopy revealed the presence of excessive lamellar inclusion bodies in Lpla2−/− alveolar and peritoneal macrophages. This foam cell phenotype is characteristic of cellular phospholipidosis (6).

Phospholipidosis is a generalized condition observed in both animals and humans that is characterized by the appearance of Lpla2+/+ mice to confirm the presence of phospholipidosis. The alveolar macrophages from the Lpla2−/− mice were markedly larger than those from the Lpla2+/+ mice. Numerous lamellar inclusion bodies, indicative of cellular phospholipidosis, were observed in the Lpla2−/− mouse alveolar macrophages (Fig. 4B). However, such lamellar inclusion bodies were only rarely present in the Lpla2+/+ cells (Fig. 4A). A similar but less robust change was also observed in the peritoneal macrophages (Fig. 4C and 4D). The increase in phospholipid accumulation corresponds to the presence of lamellar inclusions and cellular phospholipidosis.

The phospholipid content of the mouse lung and lavage fluid was measured to ascertain if the development of alveolar foam cells was associated with an increase in total phospholipid content (Table 2). No significant change in total lung phospholipid content was observed in Lpla2−/− and Lpla2+/+ 4-month-old mice. However, significant increases in both lung and lavage fluid phospholipid levels was seen in 12-month-old mice (Table 2). When the levels of unsaturated PC content were measured, an increase that was proportionate to total phospholipid levels was observed in both the Lpla2−/− mouse lung and lavage fluid.

There was no significant difference in body and organ weights between the Lpla2 genotypes at 4 months of age. A routine histological survey of their organs, including the hearts, livers, kidneys, brains, and spleen, by hematoxylin and cosin staining showed no gross differences between wild-type and homozygous mice at 4 and 12 months of age (data not shown). However, periodic acid-schiff staining of the organs revealed the presence of foam cells throughout these organs in the homozygous null mice that was most apparent in the spleens of the 1-year-old mice (Fig. 3C and D). By 12 months of age, however, significant splenomegaly was observed in the Lpla2−/− mice (Fig. 5A). A greater than fourfold increase in spleen weight was observed. Phospholipid analysis revealed that the increase in phospholipid was primarily due to changes in phosphatidylcholine and phosphatidylethanolamine, consistent with the known substrate specificity of LPLA2 (Fig. 5B and C).

**TABLE 1. ESI/MS analysis of alveolar macrophage lipids from 1-year-old mice**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Lipid levela (nmol/mg protein) by genotype</th>
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<tr>
<td></td>
<td>Lpla2+/+</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
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<td>16:0 Phosphatidylcholineb</td>
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<tr>
<td>Free fatty acid</td>
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</tbody>
</table>

a Lipid levels are derived from pooled samples of six mice (three male and three female) from each group. 16:0 designates the presence of palmitoyl containing phosphatidylcholine.

b Percent 16:0 PC for Lpla2+/+ mice, 65.0; for Lpla2−/− mice, 69.6.

c Percent plasmalogen containing phosphatidyl-ethanolamine for Lpla2+/+ mice, 69.2; for Lpla2−/− mice, 78.9.
concentric lamellar bodies within cells and the intralysosomal accumulation of phospholipids. Phospholipidosis occurs in a variety of clinical settings. Phospholipidosis may be associated with the exposure to xenobiotics. Drugs and their metabolites that induce cellular phospholipid accumulation fall into many different classes, including antiarrhythmics, antipsychotics, antibiotics, and cholesterol-lowering agents. However, these drugs share a common physiochemical structure that includes a hydrophobic ring structure and hydrophilic side chain with a charged cationic amine group (7). Phospholipidosis may also occur in response to environmental exposures such as silica (14). Finally, phospholipidosis is often used synonymously with congenital or acquired disease associated with impaired surfactant catabolism (18). Patients suffering from pulmonary alveolar proteinosis present differently from those with drug-induced phospholipidosis. Pulmonary alveolar proteinosis is characterized by a marked accumulation of surfactant lipid; drug-induced phospholipidosis is characterized by secondary inflammatory changes and long-term fibrosis. The consequences of chronic exposure to cationic amphiphilic drugs are poorly understood, in large part due to the absence of a suitable animal model for long-term evaluation (16). In particular, the relationship between the short-term development of lysosomal phospholipid accumulation and long-term inflammation and fibrosis has not been well studied. The Lpla2 knockout mouse is a potentially useful model for elucidating this relationship.

Pulmonary alveolar proteinosis may also be associated with the absence of functional surfactant protein B or loss of granulocyte-macrophage colony-stimulating factor (GM-CSF) ac-
tivity (18). Although the latter is an important factor in alveolar macrophage differentiation, the relationship between loss of GM-CSF and impaired catabolism of surfactant phospholipids is not well understood. Three histological features are observed in the lungs of the GM-CSF knockout mouse. These include the accumulation of surfactant in the airspaces, the development of a foam cell phenotype by alveolar macrophages, and a peribronchial and perivascular inflammation. Two of these findings, the foam cell phenotype and inflammation, are prominently found in the Lpla2−/− mouse. Although increased surfactant phospholipid could be detected in 1-year-old Lpla2−/− mice, this was less robust a finding than that observed in the GM-CSF knockout mouse. In prior work where Lpla2 was found to be highly expressed in alveolar macrophages, the GM-CSF knockout mouse was found to have limited expression of Lpla2, and a GM-CSF transgenic mouse was found to have increased expression of Lpla2 (1). The present study is also consistent with the possibility that Lpla2 mediates at least some of the GM-CSF-dependent changes observed in the lung.

An additional finding, not reported in the GM-CSF knockout mouse, was the presence of splenomegaly. This finding is consistent with that observed in another disorder of foam cell formation, Gaucher disease. The basis for the splenomegaly is unknown, but in a manner similar to Gaucher disease, it appears to be the result of secondary growth and not from the increase in lipid mass per se. Like Gaucher disease, this finding may be reflective of the role of the splenic macrophage in the clearance of senescent erythrocytes. The Lpla2 null mouse may

![Figure 4: Electron micrographs of alveolar macrophages (A and B) and peritoneal macrophages (C and D) obtained from 3-month-old Lpla2+/+ and Lpla2−/− mice. (A and C) Lpla2+/+ mice; (B and D) Lpla2−/− mice.]

**TABLE 2.** Protein and phospholipid content of Lpla2−/− and Lpla2+/+ mouse lung and bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Lpla2+/+</th>
<th>Lpla2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg/kg of mouse wt)</td>
<td>448.2 ± 12.9</td>
<td>525.9 ± 52.8*</td>
</tr>
<tr>
<td>Tissue</td>
<td>16.4 ± 2.6</td>
<td>16.1 ± 7.5</td>
</tr>
<tr>
<td>Lavage fluid</td>
<td>152.9 ± 155.4</td>
<td>10.7 ± 1.2**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total phospholipid (μmol/kg mouse wt)</th>
<th>Lpla2+/+</th>
<th>Lpla2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>118.9 ± 8.8</td>
<td>155.4 ± 14.9*</td>
</tr>
<tr>
<td>Lavage fluid</td>
<td>16.2 ± 1.5</td>
<td>21.6 ± 1.2**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total disaturated PC (μmol/kg mouse wt)</th>
<th>Lpla2+/+</th>
<th>Lpla2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>22.7 ± 2.2</td>
<td>35.4 ± 3.3**</td>
</tr>
<tr>
<td>Lavage fluid</td>
<td>8.3 ± 0.7</td>
<td>11.2 ± 0.7**</td>
</tr>
</tbody>
</table>

| Total                                  | 31.0 ± 2.9     | 46.6 ± 4.0**   |

* and ** denote P < 0.05 and P < 0.005, respectively (n = 4).
be a useful tool for probing the mechanism behind the lipid-induced organomegaly.

In addition to the increased accumulation of surfactant phospholipid in 1-year-old mice, including the accumulation of disaturated PC, further confirmation for a role for Lpla2 in surfactant degradation was based on the use of ESI/MS analysis of macrophages isolated from Lpla2 wild-type and null mice. These analyses revealed that of the measured lipids, PC and PE were most directly affected by the loss of Lpla2 activity. Those species of PC containing saturated fatty acids, most notably palmitate, as well as those with diacylglycerol, plasmalyl, and plasmalyn linkages increased comparably in the absence of the acidic phospholipase A2 activity. These data suggest that Lpla2 does not discriminate among PCs with different aliphatic chains. By contrast, sphingomyelin and phosphatidic acid levels were not increased, consistent with the known substrate specificity of Lpla2.

A small change in ceramide content was observed. Lpla2 was originally identified as a 1-O-acylceramide synthase (3). This increase in ceramide may reflect the loss of this activity. However, the physiological role of Lpla2 as a regulator of ceramide metabolism remains to be clarified and is not obvious from the present study. Nevertheless, Lpla2 activity was measured in the wild-type and knockout mice using both ceramide and water as acceptors for the enzyme activity. By assaying Lpla2 as either a 1-O-acylceramide synthase or as a phospholipase A2, the enzyme activity was abolished to greater than 95% in the lungs and alveolar macrophages of the knockout mice, suggesting that Lpla2 represents the primary acidic phospholipase A2 activity found in lung.

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REFERENCES


