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DRUG INDUCED PHOSPHOLIPIDOSIS: AN ACQUIRED LYSOSOMAL STORAGE DISORDER

James A. Shayman and Akira Abe

Department of Internal Medicine, University of Michigan Medical School, University of Michigan, Ann Arbor, Michigan 48109

Abstract

There is a strong association between lysosome enzyme deficiencies and monogenic disorders resulting in lysosomal storage disease. Of the more than 75 characterized lysosomal proteins, two thirds are directly linked to inherited diseases of metabolism. Only one lysosomal storage disease, Niemann-Pick disease, is associated with impaired phospholipid metabolism. However, other phospholipases are found in the lysosome but remain poorly characterized. A recent exception is lysosomal phospholipase A2 (group XV phospholipase A2). Although no inherited disorder of lysosomal phospholipid metabolism has yet been associated with a loss of function of this lipase, this enzyme may be a target for an acquired form of lysosomal storage, drug induced phospholipidosis.

Keywords

lysosome; phospholipase A2; drug induced phospholipidosis; cationic amphiphilic drug; amiodarone

1. Introduction

The discovery of the lysosome was reported by de Duve in 1955 [1]. The significance of de Duve's seminal work was immediately appreciated, and he shared the Nobel Prize in 1974. Almost 60 years later, the importance of this work remains unquestioned in no small measure due to the subsequent identification of over 50 inherited diseases resulting from the inherited loss of function of single gene products that localize to the lysosome [2]. The study of these disorders has been first and foremost valuable for the identification, management and treatment of these diseases which, although rare, collectively account for 1 in every 8000 births [3]. Of comparable importance have been the insights into the biochemistry and cellular biology of lysosomal substrates and the role of this organelle in fundamental cellular processes including autophagy, host defense, and cell death. For example, several metabolic pathways would not have been as readily identified and the focus of attention if not first identified through the characterization of these genetic diseases. Included in these pathways are those involving glycosphingolipids, mucopolysaccharides, and cholesterol esters. Insights derived from investigation of protein trafficking and vesicle sorting would not have been as readily pursued if not for de Duve's observations.

Address correspondence to: James Shayman, Department of Internal Medicine, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0676. Telephone: 734-763-0992 Fax: 734-763-0982, jshayman@umich.edu..

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In contrast to studies on these inherited disorders, considerably less attention has been focused on the concept that there may be acquired forms of lysosomal storage and that in the study of such cases additional insight into cell metabolism and cellular biology might be gleaned. This review is based on the premise that there is at least one form of acquired lysosomal storage, viz. drug induced phospholipidosis. It is argued that an understanding of drug induced phospholipidosis is not only important to the field of drug discovery and development and toxicology, but that fundamental questions surrounding drug induced phospholipidosis might be more tractable if studied in the context of lysosomal biology. In support of this premise, this review will focus on the discovery and characterization of a newly identified lysosomal enzyme, group XV phospholipase A2 [4] and its potential role as a mediator of some forms of drug induced phospholipidosis.

2. Phospholipid metabolism in the lysosome

The recognition that the lysosome was an important site for the catabolism of phospholipids was appreciated with the recognition that inherited deficiencies in acidic sphingomyelinase are the basis for Niemann-Pick disease types A and B [5]. The choline phosphohydrolase activity of this enzyme against sphingomyelin results in the formation of ceramide and phosphorylcholine. Due to the clinical importance of Niemann-Pick disease, much of the published work on lysosomal phospholipid metabolism has focused on the catabolism of sphingomyelin and less attention on more abundant phospholipids such as phosphatidylcholine and phosphatidylethanolamine. However, additional hydrolytic activities against glycerophospholipids localized to cellular lysosomal fractions and characterized by acidic pH optima do exist and were in fact first reported 45 years ago. These enzyme activities include phospholipase A1, A2, C and D.

A phospholipase A activity obtained from rat liver lysosomes was first described by Mellors and Tappel in 1967 [6]. This calcium independent enzyme had an acidic pH optimum and recognized both phosphatidylcholine and phosphatidylethanolamine as substrates. Similar phospholipase A activities in rat liver were confirmed in other reports [7, 8]. Subsequently, distinct phospholipase A1 (PLA1) and A2 (PLA2) activities were separated by gel filtration from the soluble fractions of rat liver lysosomes [9]. Additional sources of lysosomal phospholipase A activities were subsequently reported from sources that include myocardium [10], alveolar macrophages [11], arterial smooth muscle cells [12], testes [13], renal cortex [14], and peritoneal macrophages [15].

An acidic phospholipase C activity from homogenates of rat tissues including fat, brain, diaphragm, duodenum, heart, small intestine, kidney, liver, lung, skeletal muscle and spleen has also been identified [16]. In addition, lysosomal phospholipase C was identified from the soluble fractions of rat liver [17–19] and kidney [14] lysosomes, and canine cardiac sarcoplasmic reticulum [20]. The enzyme activity is characterized by has an acid pH optimum and the absence of any requirement for divalent metal ions. An uncharacterized phospholipase D activity may also be localized to lysosomes [21].

Several lysosomal PLA1s were purified from rat liver and kidney by Hostetler and colleagues. They isolated five lysosomal PLA1s with molecular weights between 20,000 and 90,000 from rat liver [22]. The differences in molecular weights between the isolated enzymes were due to distinct isoelectric points and carbohydrate moieties. Each enzyme demonstrated an acid optimum pH, the absence of a requirement of divalent metal ions, and a higher specificity toward *sn*-1 position compared to the *sn*-2 position of glycerophospholipids. The lysosomal PLA1 purified from rat kidney cortex was a glycoprotein with an isoelectric point of pH 5.4 and an apparent molecular weight of 30 kDa [23]. However, the lysosomal PLA1 gene and encoded amino acid sequence have not been

reported. It should also be noted that a lysosomal-type Ca^{2+} -independent PLA2 was isolated from rat lungs and cloned from a human myeloblast cell line [24]. The characterized enzyme was identical to peroxiredoxin 6 and is a bifunctional enzyme with activities that include glutathione peroxidase and phospholipase A2. It has been suggested that when the enzyme is located in the cytoplasm it functions as a glutathione peroxidase and when in lysosomes as a phospholipase A2 [25].

At a cellular level, there are unique obstacles that must be overcome for a phospholipase to function within the lysosome. First, the lipid substrates are present within complex membranes and must be presented to the enzyme. Second, the limiting membrane around the lysosome, although protected by a glycocalyx, is itself a potential target of the hydrolytic enzymes within the lysosome. Thus in some manner there must be separation between lysosomal enzymes and the outer lysosomal membrane. Third, the products of phospholipid and sphingolipid metabolism, including sphingosine, ceramide and lyso-phospholipids, themselves are capable of permeabilizing the lysosomal membrane, a process linked to cell death. Thus there should be some mechanism by which the accumulation of these products is limited.

Sandhoff has suggested that there are key properties shared by lysosomal hydrolases, including acid ceramidases, sphingomyelinase, and lysosomal phospholipase A2 that address these challenges [26]. First, these enzymes function within a subcompartment in the lysosome, likely against intralysosomal vesicles, where they are prevented from contacting the limiting membrane. Second, these enzymes or their associated activating proteins bind to anionic phospholipids at the acidic pH found in the lysosome. Included among these anionic lipids is BMP, a phospholipid uniquely found only the lysosome and late endosome [27]. The lysosomal lipid binding proteins include saposins or ganglioside GM2 activating protein. Third, these enzymes run their reactions in both forward and reverse preventing the reaction products from accumulating at too great a concentration [28]. Fourth, the anionic lipids to which the hydrolase bind turn over very slowly (Fig. 1).

3. BMP a phospholipid uniquely found in the late endosome/lysosome

Bis(monoacylglycero)phosphate (BMP) is an anionic phospholipid uniquely found in lysosomes and late endosome [29]. BMP has a stereoconfirmation that renders it a poor substrate for catabolism by lysosomes. BMP is negatively charged at lysosomal pH. Finally, BMP is localized to the inner membranes of lysosomes and late endosomes. BMP therefore is considered the primary anionic phospholipid that binds lysosomal hydrolases. BMP essentially consists of two monoacylglycerols bound to a single phosphate. For this reason the commonly used name for this lipid, lyso-bisphosphatidic acid, is incorrect. The resistance of BMP to phospholipases is most probably due to its *sn*-1:*sn*-1' configuration. Although some studies have suggested that this structure consists of a phospholipid with fatty acyl groups esterified groups at the *sn*-2:*sn*-2' positions. The fatty acid chains vary in composition and include oleic acid and polyunsaturated fatty acids.

The site of synthesis of BMP within cells has not been established although it may occur at the inner membrane surfaces of late endosomes and lysosomes. Given that 10 percent of BMP is in the late endosome and 90% is in the lysosome, it is reasonable to assume that synthesis occurs in one or both of these sites [30]. If this is true, then BMP would be unique among phospholipids for being synthesized within the lysosome. The synthesis of BMP within the lysosome also implies that the enzymes required for BMP synthesis should exist at these sites as well. Despite the identification of BMP more than 45 years ago, the pathway for synthesis is still not fully defined. Phosphatidylglycerol and cardiolipin have been suggested to be the precursors for de novo BMP synthesis [31]. However, a recent study in

cells deficient in phosphatidylglycerophosphate synthase, a required enzyme for phosphatidylglycerol synthesis, is consistent with phosphatidylglycerol and not cardiolipin being the precursor for BMP [32]. Waite and colleagues proposed a multi-step synthetic scheme for BMP synthesis within the lysosome based on phosphatidylglycerol as the substrate [33, 34]. They proposed that BMP synthesis requires at least three distinct steps; a phospholipase A₂ at acidic pH, the transacylation of the unesterified glycerol, and an enantiomeric rearrangement (Fig. 2).

In step one, an acidic phospholipase A₂ removes the fatty acid from position *sn*-2 of phosphatidylglycerol. In the second step, the lysophosphatidylglycerol is acylated on the *sn*-2' position of the head group glycerol moiety to yield *sn*-3:*sn*-1' bis(monacylglycero)phosphate, by means of a transacylase reaction with lysophosphatidylglycerol as both the acyl donor and acyl acceptor. It is unknown whether two separate or a single enzyme catalyzes these reactions. In step 3 there is a stereospecific conversion of the precursor molecule to the unusual *sn*-1:*sn*-1' *S*-configuration. This requires the removal of the fatty acid from position *sn*-1 of the primary glycerol unit and a rearrangement of the phosphoryl ester from the *sn*-3 to the *sn*-1 position. Waite proposed that this requires a phospholipase D. In step four, position *sn*-2 of the primary glycerol unit is esterified, probably by a transacylation reaction with another phospholipid as donor. LPLA₂, in addition to recognizing phosphatidylglycerol as substrate, has both phospholipase A₂ and transacylase activities and may potentially catalyze steps 1, 2, and 4 of this pathway.

BMP has been suggested to play two primary roles in lysosomes. One suggested role, based on the biophysical properties of BMP, is as a regulator of membrane dynamics of lysosomes [35]. This function is supported by studies that implicate BMP enriched membrane domains in invagination, vesicle formation, and fusion. A second role, as suggested above, is as a binding site for polycationic activator proteins in addition to lysosomal hydrolases. These activator proteins include saposins A through D, and GM2 activator protein. Given the critical role of these activator proteins and their associated hydrolases, any substance that interferes with this binding interaction should impair lipid catabolism within the lysosome. One class of compounds that appear to interfere with this binding interaction is cationic amphiphilic drugs. These include commonly used agents such as tricyclic antidepressants (desipramine, imipramine) [36, 37], antibiotics (azithromycin) [38], anti-arrhythmics (amiodarone) [39], and anti-malarial agents (chloroquine) [40]. There are more than fifty such agents in clinical use and they are associated with the development of lysosomal phospholipid accumulation, otherwise known as drug induced phospholipidosis (DIP).

4. Drug induced phospholipidosis

DIP was first reported in 1948 when Nelson and Fitzhugh observed the induction of foamy macrophages in rats treated with chloroquine [41]. This was later confirmed to be due to the accumulation of phospholipids within cells and documented to be a generalized response to cells exposed to cationic amphiphilic substances. Over the last 60 years over 50 drugs, many already marketed and among the most widely used in clinical practice, have been associated with phospholipidosis. With the more recent focus of the FDA on the potential toxicities of drug induced phospholipidosis, this observation has become highly problematic for the pharmaceutical industry. Many lead compounds have been dropped during development, often at late pre-clinical phases, once phospholipidosis is observed.

The pathological significance of DIP is uncertain. The affected cells are foamy in appearance and the cytoplasm is classically marked by the appearance of myeloid bodies, representing whorled membranes. Histologically this appearance is referred to as "lamellar

bodies” or “myeloid bodies”. While many cell types and organs have been reported as susceptible to DIP, there are three typical patterns of lesions [42]. The most common pattern is *macrophage dominant DIP*. Foamy alveolar macrophages, Kupffer cells, and lymphoid tissue macrophages are associated with this pattern. *Parenchymal cell dominant or generalized DIP* has also been described. Hepatocytes, renal epithelial cells, vascular endothelium, and central and peripheral nerve cells may be involved. The third pattern is referred to as *localized phospholipidosis*. This is the least common form. In this setting the susceptibility of cell types such as bile canicular cells or renal epithelial cells may reflect a particular transport mechanism for an offending drug.

The functional importance of DIP is the subject of considerable debate. The predominant view is that DIP is the basis for drug mediated cellular toxicity. In support of this view are *in vitro* studies identifying significant cellular dysfunction in the setting of DIP. These manifestations include but are not limited to impaired lysosomal protein degradation, decreased pinocytosis and endocytosis, increased free radical formation, and depressed immune responses [43–45]. A countervailing view is that DIP is an adaptive response to exposure to cationic amphiphilic drugs and is not a toxic response per se. By this theory drugs are trapped within lysosomes bound to lamellar phospholipid rich bodies and sequestered from other cellular sites where they may be toxic. The lamellar bodies are subsequently secreted by macrophages and cleared. These competing views of the pathological significance of DIP are difficult to reconcile in the absence of a clearly defined mechanism for DIP.

CADs exhibit the property of lysosomotropism, the property of a molecule to specifically associate with lysosomes. In theory, CADs could enter the lysosome through endocytosis, autophagy, or a drug-specific transporter. However, passive diffusion has been invoked as the major mechanism whereby CADs accumulate within the lysosome [46]. Lysosomotropic molecules are typically weak bases with pKa values close to or above neutrality. When unionized, CADs are membrane permeable and partition across the lysosomal membrane based on a concentration gradient. At the acidic pH of the lysosome, the lysosomotrope is converted from the free base to its ionized form. As a result, the concentration of free base within the lysosome is lowered substantially, favoring the further partitioning of drug into the lysosome. As the concentration of the ionized amine increases further, the pH of the lysosome will eventually rise toward neutrality. One consequence of this accumulation is the development of an enzymatically less favorable pH for lysosomal hydrolases [46].

In principle, there are several mechanisms by which lysosomal phospholipid content might arise in the presence of phospholipidosis inducing drugs, also known as cationic amphiphilic drugs (CADs). Sawada and coworkers proposed four potential mechanisms based on the DNA microarray expression profiles observed in HepG2 cells exposed to 12 different CADs [47]. These include decreased lysosomal enzyme transport, increased cholesterol biosynthesis, increased phospholipid biosynthesis and decreased lysosomal phospholipase activity. Unfortunately, the data in their study was limited to RNA expression measurements, and there were no correlations between the observed changes and protein expression or measurements of enzyme activity. Because phospholipid and cholesterol biosynthesis occur in cellular sites outside of the lysosome, it is difficult to postulate that these changes are the result of primary effects of CADs that localize to the lysosome. Of additional note was that the sphingolipid metabolizing enzymes acidic sphingomyelinase and acid ceramidase were observed to increase in RNA expression. Yet sphingolipid mass is infrequently observed to increase as a result of DIP. By contrast, no glycerophospholipase of any type including phospholipase A, C, or D were changed. Their findings, therefore, should be interpreted cautiously.

More direct biochemical support exists for the inhibition of a lysosomal phospholipase as the basis for DIP. Three classic CADs, chlorpromazine [48], amiodarone [49], and chloroquine [40, 50] have been reported to inhibit phospholipase A1, calcium dependent phospholipase A2, and phospholipase C activities in a dose dependent fashion. Some, but not all, of these compounds have been reported to act as competitive inhibitors of these enzymes. While these and other observations may provide intriguing clues as to the mechanism of DIP, these data are limited in two important ways. First, they do not provide an explanation for how dozens of structurally distinct CADs could act as inhibitors of the catalytic sites of one or more of these phospholipases. Second, the enzymes studied are not the phospholipases that are actually localized to the lysosome. Thus either there must be other phospholipases involved or an additional hypothesis as to how substrates that accumulate in extra-lysosomal compartments would end up in the lysosome.

Mingeot-Leclercq proposed an alternative mechanism whereby CADs could block phospholipase activity. This is known as the “negative charge hypothesis” [51]. Under this hypothesis, phospholipases interact with negatively charged phospholipids within the lysosome. The more negatively charged the membrane, the more active the phospholipase. CADs counteract this activity by binding with these membrane lipids and neutralizing the membrane charge. The attractiveness of this theory is that any lipophilic cationic amphiphile might potentially act as an enzyme inhibitor so long as the drug maintained the generalized structure of a cationic amphiphile. In other words, one would not need to postulate that CADs act in a classic manner as competitive inhibitors of a particular lipase. The inhibition of electrostatic charge interactions between lysosomal hydrolases and anionic membranes could result in the loss of the lipase activity through one of two mechanisms. The phospholipid substrate may no longer be available to the enzyme. Alternatively, the lysosomal enzyme may be displaced from its lipid binding site and itself might be a substrate for the lysosomal proteases. Sandhoff has published findings in support of the latter mechanism for the loss of sphingomyelinase activity in cells treated with tricyclic drugs [52].

The “holy grail” of research on drug induced phospholipidosis has been the search for a reliable *in vitro* or *in silico* method for predicting which compounds result in DIP. Regarding potential *in silico* techniques, structure activity relationship (SAR) profiling as a predictor of phospholipidosis has been attempted by several groups [53–60]. This work has led to the identification of similar structural properties of CADs. These properties include the presence of a hydrophobic structural domain consisting of an aromatic or aliphatic ring system. In addition, CADs typically have a hydrophilic cationic side chain with a primary, secondary, or tertiary amine. It is believed that CADs are basic drugs that cross biological membranes and once within the acidic environment of the lysosome are protonated. Once ionized, the hydrophobic core of the lysosomal membrane excludes the passage of the drug. The relative contributions of CAD structure in binding to the electrostatic and hydrophobic sites of phospholipids has been harder to discern. For example, amiodarone binds primarily to the hydrophobic moiety of phospholipids [61] whereas chlorphentermine interacts with the polar ionic moiety of phospholipids [62].

Several strategies have been employed for the empirical testing of compounds that might cause DIP. These include conventional pathological examination of tissues from animals used in toxicological studies, bronchoalveolar lavage fluid analysis of macrophages and surfactant, and *in vitro* cell assays [44, 63–66]. Cell based assays have focused on flow cytometric and morphologically based assays due to the absence of a specifically identified enzymatic target [67]. These assays typically measure cellular phospholipids with agents such as Nile red [68]. These methods are not based on an enzyme activity assay since no universally acknowledged enzyme target for CADs has been identified. An alternative

approach has been to focus on lipid metabolites that provide a signature for phospholipidosis. BMP is suggested to be one such marker [69].

The failure of prior research on phospholipidosis to identify a universal mechanism and specific target for drugs that cause lysosomal phospholipid accumulation has been consequential. First, this failure has contributed to the failure of the pharmaceutical industry and of government regulators to adequately assess the importance of this phenomenon. As such drugs in development are either dropped once observed to induce phospholipidosis or regulatory approval is delayed. Second, drugs that may be highly effective are limited in their use. Amiodarone is one such example of a potent anti-arrhythmic agent that is often not used due to phospholipidosis. On the other hand, if the mechanism for DIP were known, then potentially patients could be screened for susceptibility or resistance to DIP and these agents could be developed or if already approved more rationally administered or repurposed.

Discerning the basis for drug induced phospholipidosis presents a series of interesting and potentially important questions. These questions impact not only on practical challenges in drug development, toxicology and translational medicine, but also may lend insight into fundamental questions in phospholipid metabolism and lysosomal function. Four major questions can be considered. First, what is the mechanism by which structurally diverse compounds induce lysosomal phospholipid accumulation? Second, what are the pathological consequences of drug induced phospholipidosis? Third, can *in vitro* screening of chemical libraries be performed to predict phospholipidosis so as to shorten the pathways for drug development? Fourth, are there definable genetic factors causing phospholipidosis so that drugs can be “repurposed” to predict toxicity in susceptible populations?

Answering these questions will require a significantly better characterization understanding of the lysosomal metabolism of phospholipids. The acidic sphingomyelinase is one very well characterized lysosomal lipase. This enzyme binds to anionic phospholipids. In the presence of some CADs, the enzyme is displaced from the inner lysosomal membranes and appears to be susceptible to degradation by proteases within the lysosome [52]. However, glycerophospholipid and not sphingomyelin accumulation is commonly observed in DIP. It is reasonable, therefore, to postulate that a glycerophospholipid sensitive lipase, viz. a phospholipase A1, A2, C, or D would play a central role in DIP. A recently characterized lysosomal phospholipase A2, group XV phospholipase A2, has many of the characteristics of such a target. In the following sections the characterization, properties including substrate specificity, and role of GXV PLA2 in amiodarone induced phospholipidosis are reviewed.

5. The discovery and characterization of lysosomal phospholipase A2

A serendipitous discovery was made in 1996 during the course of studies on understanding the enantioselectivity of the glucosylceramide synthase inhibitor D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-propanol (PDMP). Glucosylceramide is catalyzed by the cerebroside synthase in the presence of ceramide and UDP-glucose. PDMP inhibits this reaction at an IC₅₀ of 20 μ M. In cell culture, the depletion in glucosylceramide is mirrored by an increase in ceramide. This was interpreted as the result of substrate accumulation. However, it was observed that while only the D-*threo* (R,R enantiomorph) was an active inhibitor of the cerebroside synthase, the three other enantiomorphs, D-*erythro*-, L-*erythro*-, and L-*threo*-, were equally active in raising cell ceramide levels [70]. A search was undertaken to identify inhibitory or activating effects of PDMP on known pathways of ceramide metabolism. No such effect on enzyme activities including sphingomyelin synthase, sphingomyelinase, galactosylceramide synthase, or neutral or acidic ceramidases could be observed.

When the homogenate of cultured MDCK cells were subsequently radiolabeled with tritiated N-acetylspingosine, the existence of a novel pathway for ceramide metabolism emerged [71]. The pathway resulted in the formation of a highly lipophilic metabolite of ceramide, acyl-ceramide. The demonstration that the 1-hydroxyl and not the 3-hydroxyl was the site of acylation was proven by converting the vicinal 2-hydroxyl of the reaction product to a ketone with 2,3-dichloro-5,6-dicyanobenzoquinone. The fatty acid donor for the formation of 1-O-acylceramide was next established. It was determined that the substrate for the reaction was a phospholipid, initially identified as phosphatidylcholine or phosphatidylethanolamine, and not free fatty acid or fatty acyl-CoA. More specifically, a fatty acid from the *sn*-2 position of PC or PE was transferred to the C-1 hydroxyl group of ceramide.

The enzyme was purified to homogeneity from bovine brain. Upon purification of the enzyme, it was determined that either ceramide or water could serve as acceptors for the *sn*-2 fatty acid (Fig.3)[72]. In the presence of water alone, the enzyme displayed properties of a classic phospholipase A2. The bovine purified phospholipase was also demonstrated to have broad specificity, recognizing phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine. Two characteristics of this phospholipase A2 immediately became apparent. First, the enzyme displayed an acidic pH optimum; second, the enzyme localized to lysosomes. Based on the enzyme purification, several partial amino acid sequences of LPLA2 were determined and the full length coding and full length amino acid sequences deduced [73]. The expressed protein was confirmed to have both phospholipase A2 and transacylase activities. Analysis of the coding sequence revealed that LPLA2 is 49% identical at the amino acid level with lecithin cholesterol acyltransferase (LCAT). Most of the identity was localized to the catalytic domain. Both LCAT and LPLA2 both localized to 16q22.1 where they are contiguously placed, consistent with one gene arising from a duplication event. Phylogenetic analysis revealed that both genes are derived from diacylglycerol acyltransferase and that LPLA2 is the ancestral precursor to LCAT.

A more detailed analysis of the catalytic domain revealed that LPLA2 contains a consensus sequence, -A-X-S-X-G- that is similar to the -G-X-S-X-G- motif present in other phospholipase A2s and acylhydrolases (Fig. 4) [74]. The -A-X-S-X-G- motif is found in a lipase of *Bacillus subtilis*. The serine is part of a catalytic triad composed of an asparagine and histidine residue in close approximation and serving to convert the serine into a strong nucleophile. Site directed mutagenesis of the triad amino acids eliminated the intrinsic phospholipase and transacylase activities of LPLA2. Additional analysis revealed the presence of a single disulfide bridge between Cys65 and Cys89 [74]. The *sn*-1 versus *sn*-2 specificity of the enzyme was subsequently assessed. LPLA2 displays high *sn*-2 positional specificity toward phospholipids. However, this specificity changes to *sn*-1 in phosphatidylcholine when arachidonate is in the *sn*-2 position [75]. Other lipophilic alcohols besides ceramide can serve as acceptors for the fatty acid via the transacylation activity [76]. Based on the unique features of LPLA2, the enzyme has recently been designated as the first member of group XV phospholipase A2s [77].

The tissue and cellular expression of LPLA2 was also measured. LPLA2 activity was readily detectable in every organ assayed. Remarkably, however, when alveolar macrophages were isolated and compared to peritoneal macrophages and circulating monocytes, the LPLA2 activity was noted to be approximately 50 fold higher compared to other cells. This was confirmed by real time polymerase chain reaction measurements of LPLA2 RNA levels when compared to other candidate phospholipase A2s [78].

6. Creation and phenotypic characterization of Lpla2 null mice

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 phospholipase A2 activity [79]. 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 PC. By three months of age, 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. The ultrastructural examination of Lpla2^{-/-} mouse alveolar and peritoneal macrophages revealed the appearance of foam cells with lamellar inclusion bodies, a hallmark of cellular phospholipidosis. This finding was not surprising since very high expression of Lpla2 mRNA and protein had been observed in alveolar macrophages [78]. By six months of age the Lpla2 null mice began to develop signs of lymphoproliferation. Splenic enlargement was apparent as was lymphadenopathy. By 18 months of age the spleens were greater than 10 times the weight of those obtained from the wild type mice and diffuse lymphadenopathy was readily apparent. Female mice demonstrated a significantly greater predilection toward increased organ weight than male mice. By 20 months of age the knockout mice displayed weight loss with signs of wasting as evidenced by loss of subcutaneous fat. Histological analysis of the lymphoid organs revealed the presence of macrophages with a foam cell phenotype and the loss of stromal architecture.

The conversion of the knockout mouse alveolar macrophages to a foam cell phenotype, in association with an increase in the phosphatidylcholine and phosphatidylethanolamine content of the surfactant obtained from bronchoalveolar lavage, was consistent with a role for LPLA2 in surfactant catabolism. The foam cell phenotype was also consistent with the histological changes that accompany long term toxicity associated with the anti-arrhythmic drug amiodarone. This finding led to further studies to determine if LPLA2 was a specific target for CADs of this type.

7. Cationic amphiphilic drugs inhibit LPLA2 activity and result in cellular phospholipidosis

The original clue to the existence of LPLA2 was the observation that PDMP inhibited an acidic transacylation of ceramide localized to the lysosomal fraction of cells. Recently, we have revisited the question as to the basis for this inhibition. The recognition that PDMP is structurally similar to drugs that are chemically characterized as cationic amphiphilic drugs raised the possibility that other drugs in this class might be causing cellular phospholipidosis through a comparable mechanism. To test this possibility, MDCK cells were employed comparing PDMP, amiodarone, and tetracycline [80]. Amiodarone is a prototypic agent that causes phospholipidosis; tetracycline has no known association with phospholipidosis. A concentration dependent inhibition of LPLA2 activity was observed in the presence of PDMP and amiodarone, an effect not observed with tetracycline (Fig 5). MDCK cells were next treated for 1 or seven days with PDMP, amiodarone, or tetracycline. Amiodarone treatment also resulted in a time dependent increase in cell phospholipids. Notably increased were PC and PE the primary substrates for LPLA2. Of the other glycerophospholipids, phosphatidylserine was also increased but phosphatidylinositol was not. Additionally, no change in sphingomyelin was observed. These changes were consistent with the known substrate specificity of LPLA2.

To better understand the mechanism whereby LPLA2 was being inhibited by amiodarone, the ceramide transacylation reaction based on the formation of 1-*O*-acyl-N-acetylsphingosine in the presence of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine containing liposomes was conducted in the presence of a series of anionic phospholipids [81]. Dioleoyl forms of phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, and phosphatidylethanol were all potent activators of LPLA2. When BMP was substituted in the reaction, the transacylation of ceramide was even greater. However, the greatest activity was observed in the presence of dioleoyl-phosphatidylethanol.

These findings raised the possibility that amiodarone and PDMP, two structurally distinct drugs with different mechanism of action, were inhibiting LPLA2 activity by interfering with the electrostatic charge interaction between the enzyme and the liposomal membranes containing the phospholipid substrate. To test this hypothesis, LPLA2 activity measurements were compared in assays with liposomes containing dioleoyl-PC with or without the non-hydrolyzable sphingolipid sulfatide (Figs. 6A and B). The LPLA2 reaction rate was significantly greater in the presence of sulfatide. To control for the addition of a sphingolipid to the liposomes, the LPLA2 reaction was also performed with liposomes containing galactosylceramide in place of sulfatide (Fig. 6C).

To test the importance of electrostatic charge interactions in LPLA2 activity, the transacylation reaction was repeated under conditions of increased ionic strength and varying pH. LPLA2 activity was markedly lowered when the NaCl concentration was greater than 200 mM and the pH was greater than 5.5. Importantly, the pre-incorporation of amiodarone into the liposomes also inhibited LPLA2 at concentrations consistent with those observed resulting in cell phospholipid accumulation.

The esterase activity of LPLA2 was observed to be present against the non-lipophilic, water soluble substrate *p*-nitro-phenylbutyrate [81]. If the hydrolysis of glycerophospholipids embedded in membranes or assayed in liposomes required an electrostatic charge interaction with anionic lipids, then it was reasoned that the effects of pH and ionic strength would not be observed with this substrate. Indeed, when *p*-nitro-phenylbutyrate was substituted for dioleoyl-phosphatidylcholine containing liposomes, the effects of pH and ionic strength were no longer seen. Unlike the transacylase activity, the esterase activity at neutral pH was approximately 60 percent of that observed at acidic pH and was not significantly inhibited by the addition of amiodarone or NaCl.

Based on these observations, a model for LPLA2 binding to lipid membranes was considered. In this model, cationic residues on the hydrophilic surface of LPLA2 bind to anion phospholipids present in membranes that also contain the LPLA2 substrate. Amiodarone and perhaps other CADs incorporate into the membranes lowering the net surface charge and the binding affinity of LPLA2 for the membrane. One prediction from this model is that raising the pH of the reaction will inhibit LPLA2 activity by impairing the electrostatic charge interaction between the enzyme and membrane. In support of this model is that when an artificial water soluble substrate, *p*-nitro-phenylbutyrate, was used in the LPLA2 reaction, the pH dependency was lost.

To test this model, evidence for the existence of a direct physical association between LPLA2 and anionic phospholipids was sought. Recombinant LPLA2 was co-sedimented in the presence of liposomes, separated by SDS-polyacrylamide electrophoresis, and stained under a variety of incubation conditions. As predicted, liposomes containing sulfatide but not galactosylceramide were associated with a significantly greater recovery of enzyme. Increased pH, higher ionic strength and amiodarone each significantly decreased the ability of LPLA2 to co-sediment with the sulfatide-enriched liposomes.

These results are consistent with the following model of amiodarone associated inhibition of lysosomal phospholipase activity (Fig. 7). In this model, LPLA2 is bound to the inner membrane of the lysosome through electrostatic charge interactions between cationic residues found on the lipase and anionic phospholipids, including BMP, associated with the lysosome. As amiodarone accumulates within the lysosome, the drug is incorporated into lysosomal membranes resulting in a less negatively charged membrane and the dissociation of LPLA2 from its membrane binding. The dissociated lipase not only loses its ability to bind its membrane associated substrates, but is now itself susceptible to lysosomal proteases. The co-sedimentation and enzyme activity measurements provide experimental support for the membrane binding aspects of this model. The degradation of unbound LPLA2 has not yet been measured and is only postulated at this point.

8. Unanswered questions and future directions

How many lysosomal enzymes regulating PL metabolism have yet to be identified and specifically how many function in phospholipid catabolism? While GXVPLA2 represents a broad specificity phospholipase, there are phospholipids that are not substrates (e.g. BMP and PI). Phospholipase activities (e.g. PLC, PLD, and PLA1) have been reported with acid pH optima, but to date no corresponding genes or protein sequences have been reported. An ancillary question is whether BMP synthesis is catalyzed by GXVPLA2. Waite predicted that an acidic PLA2 and a transacylase are required for BMP synthesis [34]. LPLA2 is characterized by both of these activities as well as the ability to use phosphatidylglycerol as a substrate. A role for LPLA2 or as yet uncharacterized PLA2 in BMP synthesis remains to be established. The tools for discovery of these presumptive enzymes are available and they include traditional protein purification, transcriptomic, and proteomic strategies. It can be argued that a focused effort to identify and characterize these additional lipases will at a minimum result in the identification of new and perhaps more specific targets for CADs.

The ability to demonstrate a causal nexus between DIP for a particular agent and the loss of function of a lipid metabolizing enzyme will have two potentially important benefits. First, it may lead to a better understanding of the toxicology of phospholipidosis from studies linking loss of the enzyme's function with a pathological phenotype. The LPLA2 knockout mouse provides a model that represents the loss of a major lysosomal lipase and may provide a roadmap for the evaluation of the short and long term consequences of lysosomal phospholipidosis. In addition, by the identification of new targets for CAD toxicity, it may be possible to screen chemical libraries generally and drug candidates more specifically for their ability to inhibit these enzymes, creating a more efficient pipeline for drug development. Second, better mechanistic insights may allow certain agents to be administered in a more "personalized" manner in that the population at risk may be defined by genetic variability in the expression or activity of the enzyme. Individuals with high residual enzyme activities may represent those with a high therapeutic index for exposure to the drug. Those who carry polymorphisms or that result in diminished activity may be highly susceptible to phospholipidosis and thus identified as individuals who should not receive such an agent. In this manner, drugs with high clinical efficacy but with unacceptable toxicological profiles, such as amiodarone, might be repurposed after a population at risk is screened.

Finally, with the discovery and characterization of each novel lysosomal protein, one may ask is whether or not there is a previously unknown clinical disease associated with a loss of function of that enzyme or transporter. There are currently about 80 lysosomal proteins that have been identified and characterized and yet there are fifty clinically important, albeit rare diseases associated with a loss of function. A reasonable prediction is that there are new

diseases yet to be discovered and that one strategy for doing so would be to more fully characterize those genes and their encoded proteins with roles in lysosomal biology.

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Abbreviations

BMP	bis(monoacylglycero)phosphate
CAD	cationic amphiphilic drug
DIP	drug induced phospholipidosis
GXVPLA2	group XV phospholipase A2
LPLA2	lysosomal phospholipase A2
MDCK	Madin Darby canine kidney
PDMP	D- <i>threo</i> -1-phenyl-2-decanoylamino-3-morpholino-propanol

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Highlights

- Lysosomal storage diseases may be either inherited or acquired.
- Drug induced phospholipidosis is a form of acquired lysosomal storage disease.
- The mechanism for and pathological significance of phospholipidosis is not well understood.
- LPLA2 is a recently characterized lysosomal hydrolase.
- LPLA2 is a target for amiodarone induced phospholipidosis.

Figure 1.

Common properties of lysosomal hydrolases. (1) There is subcompartmentalization leading to separation from the limiting membrane. The hydrolases are active against intralysosomal vesicles that contain the substrates for these enzymes. (2) A binding interaction occurs between the enzyme and the intralysosomal membrane at acidic pH. (3) The hydrolases catalyze reactions reversibly. (4) The anionic phospholipids within the membranes such as BMP and phosphatidylinositol are slowly metabolized.

Figure 2.

The synthesis of BMP. The pathway as proposed by Waite consists of four steps. These include the hydrolysis of the *sn*-2 fatty acyl group from phosphatidylglycerol by an acidic phospholipase A2, the transacylation of the *sn*-1' hydroxyl, the reorientation of the phosphoryl ester from the *sn*-3 to *sn*-1 position, and the transacylation of the original glycerol backbone.

Figure 3.

Dual activities of lysosomal phospholipase A2 (GXVPLA2). In the presence of N-acetylseringosine, the enzyme is both an acidic phospholipase A2 and transacylase of the 1-hydroxyl group. When only water is present as an acceptor, only phospholipase A2 activity is observed.

1-60	MGLHLRPYRVGLLPDGLLFLLLLMLLADPALPAGRHPVVLVPGDLGNQLEAKLDKPTV
61-120	VHYLCSKKTESYFTIWLNLLELLLPVIIDCWIDNIRLVYNKTSRATQFPDGVDRVPGFGK
121-180	TFSLEFLDPKSSVGSYFHTMVESLVGWGYTRGEDVRGAPYDWRRAPNENGPYFLALREM
181-240	IEEMYQLYGGPVVLVAHSMGNMYTLYFLQRQPQAWKDKYIRAFVSLGAPWGGVAKTLRVL
241-300	ASGDNNRIPVIGPLKIREQQRSVSTSWLLPYNTWSPEKVFVQTPTINYTLRDYRKKFQ
301-360	DIGFEDGWLMRQDTEGLVEATMPPGVQLHCLYGTGVPTPDSFYYESFPDRDPKICFGDGD
361-412	GTVNLKSALQCQAWQSRQEHQVLLQELPGSEHIEMLANATTLAYLKRVLG

Figure 4.

The amino acid sequence of lysosomal phospholipase A2. The signal peptide sequence is highlighted in yellow. The catalytic domain that is shared with lecithin cholesterol acyltransferase is underlined in purple. The catalytic serine and its associated consensus sequence are highlighted in green as are other amino acids comprising the catalytic triad. Finally, glycosylation sites are highlighted in blue.

Figure 5. Inhibition of lysosomal phospholipase activity by amiodarone and PDMP. LPLA2 activity was measured as the formation of 1-O-acylceramide in the presence of varying concentrations of amiodarone, tetracycline, and PDMP. The cationic amphiphiles amiodarone and PDMP inhibited the transacylation reaction but tetracycline did not. This figure is reprinted from reference [80].

Figure 6.

Role of anionic lipids in the transacylation of N-acetyl sphingosine by lysosomal phospholipase A2. The reaction mixture contained 48 mM sodium citrate (pH 4.5), 10 μg/ml BSA, liposomes (127 μM phospholipid), and 14.5 ng/ml of recombinant mouse LPLA2 in 500 μl of total volume. A: Liposomes consisting of DOPC-NAS (3:1, molar ratio) or DOPC-sulfatide-NAS (3:0.3:1, molar ratio) were incubated with the enzyme for 5, 10, 15, 30, and 45 min at 37°C. The reaction products were extracted and separated by an HPTLC plate using a solvent system consisting of chloroform-acetic acid (9:1, v/v). The reaction product, 1-O-oleoyl-NAS, quantified by scanning the plate, was plotted against time (B). C: Liposomes consisting of DOPC-galactosylceramide-NAS or DOPC-sulfatide-NAS with a different molar ratio were incubated with the recombinant enzyme at 37°C. The reaction products were extracted and separated as described in A. Error bars indicate SD. (n = 3). This figure is reprinted with permission from reference [81].

Figure 7.

Proposed model for the inhibition of lysosomal phospholipase A2 and induction of phospholipidosis by amiodarone. Under normal conditions, lysosomal phospholipase A2 is bound through electrostatic charge interactions to the inner membranes of the lysosome. Amiodarone, a cationic amphiphile, accumulates in the lysosome and inserts into the inner membrane decreasing or eliminating the anionic binding sites for the lipase. The lipase is no longer able to bind to its substrates and is potentially susceptible to degradation by lysosomal proteases.