

A Role for Lysosomal Phospholipase A2 in Drug Induced Phospholipidosis

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Abstract: Many therapeutic drugs currently in use are cationic amphiphiles. These cationic amphiphilic drugs (CADs) induce phospholipidosis in humans and experimental animals. The recent study shows that CAD-induced cellular phospholipidosis is linked to the impairment of phospholipid catabolism by inhibition of lysosomal phospholipase A2 activity.

Key Words: Cationic amphiphilic drug, amiodarone, PDMP, tetracycline, phospholipidosis, lysosomal phospholipase A2.

INTRODUCTION

CADs are lipophilic amine compounds that share distinctive physicochemical structures, including a hydrophobic domain and a hydrophilic domain with an ionizable amine group which becomes positively charged under physiological conditions [1]. In general, CADs accumulate in lysosomes and perturb lysosomal enzyme activities. Some CADs induce an inhibition of phospholipid catabolism in lysosomes and increase cellular phospholipid in human and other mammalian tissues. A massive accumulation of phospholipid in the cell leads to the formation of numerous multi-lamellar inclusion bodies in cell cytoplasm resulting in loss of cellular functions and viability. Several CADs are in clinical use, including amiodarone (AMIOD), prehexiline, fluoxetine, gentamicin and 4,4'-diethylaminohexestrol, all of which have associated phospholipidosis in humans [1].

The lung is particularly sensitive to the effects of CADs. AMIOD is an extensively used anti-arrhythmic and that has been well established to promote pulmonary pathology and toxicity [2, 3, 4]. AMIOD-associated pulmonary toxicity is characterized by phospholipidosis in the lung with extensive phospholipid storage in alveolar macrophages (AM) and alveolar type II cells [2, 5, 6, 7]. AM are the most significantly affected cell-type in the setting of AMIOD induced phospholipidosis [8]. CAD-induced phospholipidosis linked to pulmonary toxicity has been proposed to result from the inhibition of lysosomal phospholipase A [9, 10, 11].

A decade ago, a novel enzyme was found, which possesses both transacylase and phospholipase A2 enzyme activities under acidic conditions, in the soluble fraction of Madin-Darby canine kidney (MDCK) cells [12]. The enzyme was subsequently purified from bovine brain and shown to be a water-soluble glycoprotein consisting of a single polypeptide chain with a molecular weight of 45 kDa. The Ca²⁺-independent phospholipase A2 activity displayed a pH optimum of 4.5 [13]. Partial amino acid sequences were obtained from the purified enzyme, and the bovine, mouse and human genes coding the enzyme were cloned [14]. The

enzyme primarily localizes to lysosomes [14]. Therefore, the enzyme was named lysosomal phospholipase A2 (LPLA2). A recent study showed that LPLA2 in mouse and rat is highly expressed in AM and may be involved in the catabolism of phospholipid in alveolar constituents such as pulmonary surfactant [15]. Additionally, AM and peritoneal macrophages (PM) obtained from LPLA2 deficient mice show a significant accumulation of phospholipid and formation of numerous multi-lamellar inclusion bodies in their cytoplasm, which is a hallmark of cellular phospholipidosis [16]. These results indicate that the impairment of LPLA2 activity may be associated with CAD-induced cellular phospholipidosis.

To evaluate whether LPLA2 is involved in the phospholipidosis induced by CADs, we investigated the effects of three CADs, AMIOD, *D-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and tetracycline, on MDCK cells and LPLA2 activity. Hostetler has reported that MDCK cells suffer from phospholipidosis after certain CAD treatment and proposed a three-step mechanism in the induction of cellular phospholipidosis by CADs: 1) the CADs enter the target cell readily; 2) they concentrate in cellular lysosomes; and 3) they inhibit lysosomal phospholipases [17]. The present short review discusses how LPLA2 is involved in cellular phospholipidosis induced by some CADs.

1. EFFECT OF CATIONIC AMPHIPHILIC DRUGS ON PHOSPHOLIPID ACCUMULATION IN MDCK CELLS

1. 1. Amiodarone (AMIOD)

AMIOD is a well-known anti-arrhythmic drug and is used in the treatment of a wide range of cardiac tachyarrhythmias, including both ventricular and supraventricular arrhythmias. Although it is widely used as an anti-arrhythmic, AMIOD causes numerous untoward effects [18]. The most serious side effect is pulmonary toxicity associated with the accumulation of phospholipid in lungs [2, 3, 4]. Phospholipid accumulation induced by AMIOD or other CADs has been shown to occur in many different types of cells. For example, AM as well as MDCK cells are affected by AMIOD. Cultured AM isolated from wild-type rats were treated with 10 μ M AMIOD, resulting in cellular phospholipidosis [19]. Hostetler reported that various CADs cause phospholipidosis in MDCK cells [17]. Recently, a significant accumulation of phospholipid was observed in MDCK cells treated with 15 μ M AMIOD. As shown in Fig. (1), the phospholipid content

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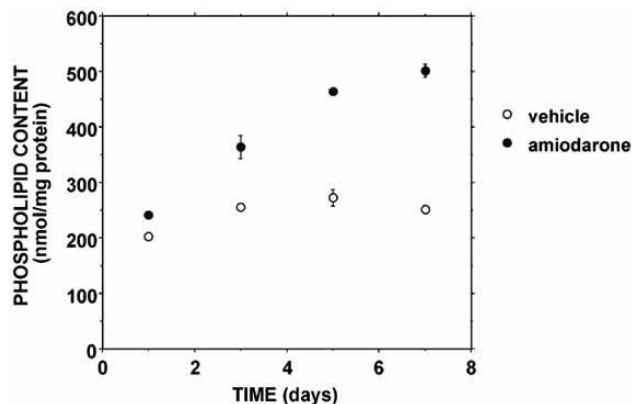


Fig. (1). Accumulation of phospholipid in AMIOD-treated MDCK cells.

MDCK cells were treated with or without 15 μ M AMIOD for 1, 3, 5 and 7 days. The medium was replaced every other day with 2 ml of fresh DMEM-F12 supplemented with 10% FCS and antibiotics (penicillin-streptomycin) plus or minus AMIOD. After the treatment, the cells were collected and cellular lipids were extracted. Phosphate content in each lipid extract was determined by the method of Ames [31]. Closed circle and open circle show with and without AMIOD, respectively. Error bar indicates S. D. (n=3).

in AMIOD-treated MDCK cells increased in a time dependent manner. After AMIOD treatment for 7 days, the phospholipid content of the AMIOD-treated cells was twice as high as that of the untreated cells. More specifically, a marked accumulation of phosphatidylethanolamine (PE), phosphatidylserine (PS) plus phosphatidylinositol (PI) and phosphatidylcholine (PC) occurred in a time dependent manner in the AMIOD-treated MDCK cells (Fig. (2)). However, there were no significant differences in sphingomyelin (SM) level be-

tween AMIOD-treated and untreated cells over the time-course.

1. 2. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP)

PDMP, which was designed as an analog of glucosylceramide, is a potent inhibitor of glucosylceramide synthase [20]. Rosenwald and Pagano previously reported that a high concentration PDMP (50 μ M) causes phospholipidosis in CHO cells [21]. CHO cells, however, are not the only cell type affected by PDMP in this way. When MDCK cells were treated with 15 μ M PDMP for 7 days, the phospholipid content in the PDMP-treated cells slightly, but significantly, increased compared with that in the untreated cells. However, when MDCK cells were treated with 50 μ M PDMP for 5 days, the total phospholipid content and phospholipid profile of the PDMP-treated cells resembled that of the AMIOD-treated cells (Fig. (3)).

2. ULTRASTRUCTURE OF AMIOD-TREATED AND PDMP-TREATED MDCK CELLS

In cellular phospholipidosis induced by CADs, the cells contain multiple lamellar inclusion bodies, which are lysosomal in origin. These cytoplasmic inclusion bodies consist primarily of accumulated phospholipids that result from impaired phospholipid catabolism after autophagy or heterophagy. AMIOD and PDMP are weak bases and have an affinity for acidic compartments in cells. They are lysosomotropic reagents that are trapped in lysosomes of the cells and affect lysosomal enzyme activities directly or indirectly. Histology was performed in MDCK cells treated with 15 μ M AMIOD or 50 μ M PDMP for 5 days. The increased phospholipid accumulation was associated with numerous lamellar bodies in the cytoplasm of the PDMP-treated cells as well

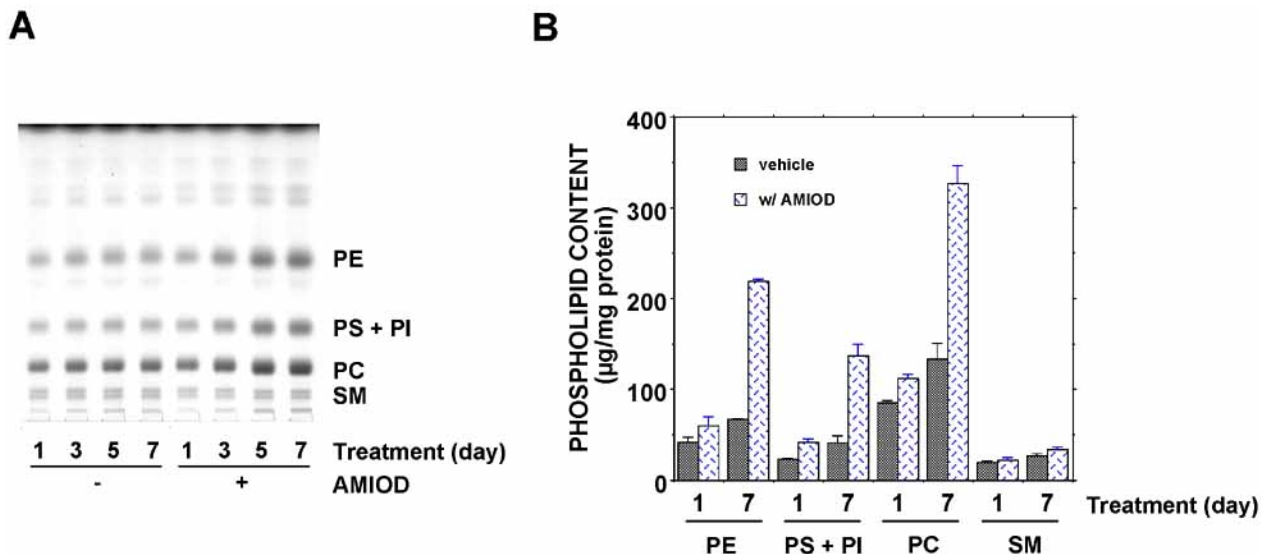


Fig. (2). Phospholipid profile in AMIOD-treated MDCK cells.

Lipid extracts obtained in Fig. 1 were applied to HPTLC plates. A phospholipid amount corresponding to 30 μ g of protein of cell homogenate was applied to each lane and developed in a solvent system consisting of chloroform/methanol/acetone/acetic acid/water (40:15:15:10:2, v/v). The phospholipids were visualized by charring with phosphoric acid/copper sulfate and scanned. Panel A shows thin layer chromatography. PE, PS, PC, PI and SM indicate phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidylinositol and sphingomyelin, respectively. Panel B shows the content of the various phospholipid groups on day 1 and day 7. Error bar indicates S. D. (n=3).

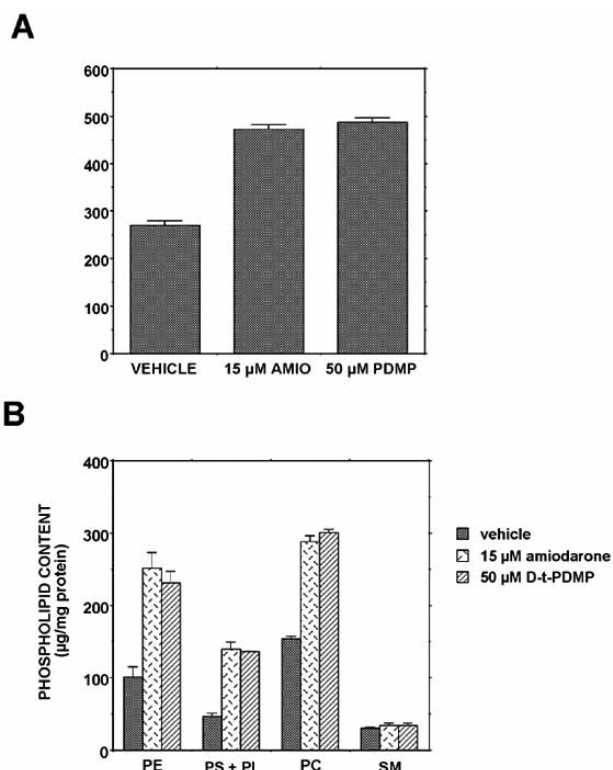


Fig. (3). Accumulation of phospholipid in PDMP-treated MDCK cells.

MDCK cells were treated with or without 15 µM AMIOD or 50 µM PDMP for 5 days. After the treatment, the cells were collected and cellular lipids extracted. Phospholipid content and phospholipid profile in each group is shown in A and B, respectively. Error bar indicates S. D. (n=3).

as AMIOD-treated cells, as revealed by electron microscopy (Fig. (4)). The formation of multi-lamellar bodies in AMIOD- and PDMP-treated MDCK cells is distinctive of phospholipidosis and may be due to a decreased phospholipid catabolism in lysosomes through the inhibition of lysosomal phospholipase activity by AMIOD and PDMP.

3. EFFECTS OF AMIOD, PDMP AND TETRACYCLINE ON LPLA2 ACTIVITY

Lysosomal phospholipase A plays an important role in lysosomal glycerophospholipid catabolism in mammalian cells. Although there have been various reports demonstrating CADs inhibit lysosomal phospholipase A, the studies have not been able to definitively distinguish the effects of the drugs on phospholipase A2 (PLA2) from that of phospholipase A1 (PLA1) because of the use of crude cell extracts as the enzyme source. Recently, the gene encoding lysosomal phospholipase A2 (LPLA2) that is highly conserved between mammals was cloned [14, 15]. To evaluate the effect of CADs on LPLA2, the soluble fraction obtained from LPLA2-overexpressing COS-7 cells was used as the enzyme source. Additionally, to confirm that the CADs affected LPLA2 activity specifically, the LPLA2 activity was assayed as phospholipid-ceramide transacylase activity in the soluble fraction under acidic conditions [12, 13, 14, 15].

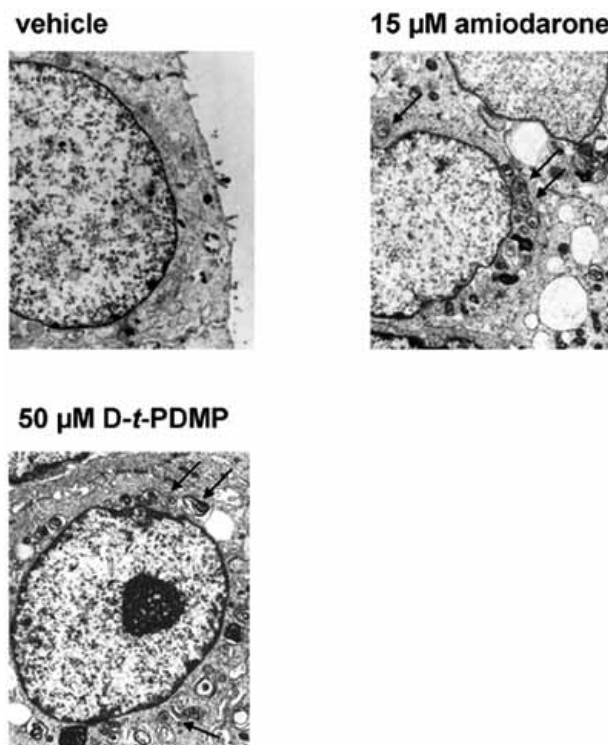


Fig. (4). Electron micrographs of MDCK cells.

Electron micrographs of MDCK cells treated with or without 15 µM AMIOD or 50 µM PDMP for 5 days. Arrows in B and C show lamellar bodies.

AMIOD and PDMP inhibited the transacylase activity of LPLA2 in a concentration dependent manner (Fig. 5). The IC_{50} s of AMIOD and PDMP for LPLA2 activity were 15 µM and 40 µM, respectively. In addition, the log *P*, 1-octanol-water partition coefficient, is 7.954 and 3.709 for AMIOD and PDMP, respectively [22]. The log *P* is an inherent parameter of the hydrophobicity for each compound. Therefore, AMIOD is a more lipophilic molecule than PDMP, suggesting that the inhibition of LPLA2 activity by CAD is dependent upon the hydrophobicity of CAD.

Tetracycline is a broad-spectrum antibiotic produced by the streptomycetes bacterium and is used against many bacterial infections. Tetracycline is a CAD but a less amphiphilic molecule (log *P*: -0.646) than AMIOD (7.954) and PDMP (3.709). There has been no report that tetracycline induces phospholipidosis. As expected, unlike AMIOD and PDMP, when MDCK cells were treated with 15 and 50 µM tetracycline for 5 days, tetracycline failed to induce an accumulation of phospholipid in MDCK cells. Additionally, tetracycline did not have any effect on LPLA2 activity, even at 300 µM (Fig. (5)). These results indicate that the ability of CADs to induce cellular phospholipidosis may be associated with CADs' hydrophobicity and ability to inhibit LPLA2 activity.

The inhibition of phospholipid degradation by phospholipase A, A1 and A2, in lysosomes in CAD-treated cells has been proposed to result from formation of indigestible phospholipid-CAD complexes [23], or direct interaction of lysosomal phospholipase A by CADs [24]. CADs with a positive log *P* value such as AMIOD and PDMP have higher

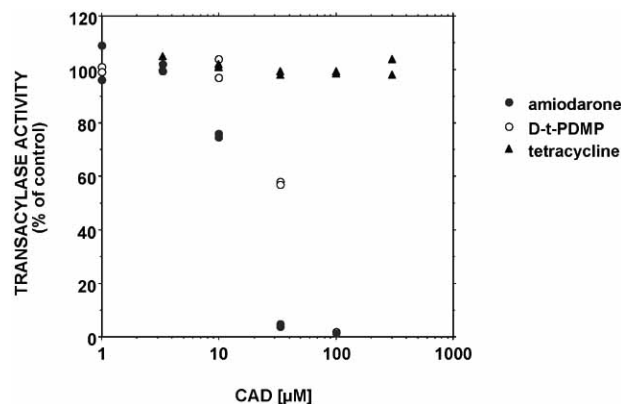


Fig. (5). Effect of CAD on LPLA2 activity.

The soluble fraction (2 μg of protein) obtained from LPLA2-overexpressing COS 7 cells was incubated for a suitable time period at 37°C in citrate buffer, pH 4.5, with 40 μM *N*-acetylsphingosine (NAS) incorporated into phospholipid liposomes (PC/PE/dicetyl phosphate/NAS (5:2:1:2 in molar ratio)), and formation rate of 1-*O*-acyl-NAS was determined. Closed circle, open circle and closed triangle indicate AMIOD, PDMP and tetracycline, respectively.

affinity and permeability to lipid membranes than polar CADs such as tetracycline. This might indicate that apparent inhibition of LPLA2 activity by the CADs is due to formation of indigestible phospholipid-CAD complexes. Taken together, the extent of lipophilicity of a CAD may be an important factor in CAD-induced phospholipidosis that is associated with inhibition of LPLA2. To further demonstrate this concept, more detailed, systematic studies using other CADs need to be performed.

4. LPLA2 DEFICIENT MICE

LPLA2 is highly expressed in AM of rats and mice [15]. LPLA2 expression in GM-CSF deficient mice, a model of impaired surfactant catabolism, is extremely low compared with wild type mice [15]. This observation suggests that LPLA2 plays a significant role in the lysosomal degradation of phospholipids. Recently, LPLA2 deficient mice were created and found that the deficiency induces phospholipidosis in AM and PM from these mice at an early age [16]. Such pronounced accumulation of phospholipid, particularly in AM, may be because phagocytic cells actively ingest foreign materials containing phospholipids such as surfactant, lipoproteins and microorganisms. PE and PC are the major phospholipids that accumulate in LPLA2 knockout mouse macrophages, whereas PS, SM and PI levels are not affected in LPLA2 deficient mice [16]. This pattern of accumulated phospholipid is consistent with the substrate specificity of LPLA2 [12]. Therefore, the accumulation of PE and PC in macrophages results from the impairment of phospholipid degradation by the deficiency of LPLA2 and leads to phospholipidosis. More recently, it has been demonstrated that LPLA2 has both PLA1 and PLA2 activities, and that the deacylation of PC and PE in lysosomes of mouse AM is mostly due to LPLA2 [25].

The recent observations in LPLA2 knockout mice and CAD-treated MDCK cells indicate the interpretation that the

selective accumulation of phospholipid (primarily PE and PC) and the cellular phospholipidosis induced by certain CADs are relevant to the impairment of phospholipid catabolism through the inhibition of LPLA2 activity.

CONCLUDING REMARKS

Significant evidence suggests that LPLA2 is involved in the phospholipidosis induced by CADs. AMIOD and PDMP have been shown to induce the accumulation of phospholipid and formation of numerous multi-lamellar inclusion bodies, both of which are distinctive for phospholipidosis in MDCK cells. Compared to AMIOD, a higher concentration of PDMP was required to induce such lipid storage disorder in MDCK cells. The IC₅₀ of PDMP for LPLA2 activity was three times higher than that of AMIOD. Unlike AMIOD or PDMP, tetracycline had no effect on phospholipid accumulation in MDCK cells or LPLA2 activity. Interestingly, it was found that the major components of the accumulated phospholipid in AMIOD- and PDMP-treated MDCK cells were PE, PS + PI and PC but not SM. LPLA2 has the substrate specificity toward PE and PC [12]. There have been reports for a stimulatory effect of compounds belonging to CADs on PS synthesis [26, 27, 28, 29, 30]. Taken together, these results indicate that certain CADs contribute to the selective accumulation of phospholipids and phospholipidosis by inhibiting LPLA2 activity.

AM and PM prepared from LPLA2 deficient mice show the selective accumulation of phospholipid and development of phospholipidosis as seen in MDCK cells treated with AMIOD and PDMP. Thus, the cellular phospholipidosis induced by CADs is associated with the inhibition of LPLA2 activity by CADs. Because of its role in phospholipidosis, LPLA2 may be a promising target to screen the potential for newly or already developed CADs to induce cellular phospholipidosis.

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ABBREVIATIONS

CAD	= Cationic amphiphilic drug
AMIOD	= Amiodarone
LPLA2	= Lysosomal phospholipase A2
PDMP	= <i>D</i> -threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
AM	= Alveolar macrophages
PM	= Peritoneal macrophages
MDCK	= Madin-Darby canine kidney
CHO	= Chinese hamster ovary
PE	= Phosphatidylethanolamine
PS	= Phosphatidylserine
PC	= Phosphatidylcholine

PI = Phosphatidylinositol

SM = Sphingomyelin

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