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Cellular Phospholipid - Accumulation Induced by Basic Drugs does not depend on Phospholipid Uptake nor Neutralization of Acidic Vesicles

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Abstract

Objectives : Drug induced-phospholipidosis is a pathological state caused by repeated administration of cationic amphiphilic drugs. The mechanism of drug-induced phospholipidosis is unclear. Several hypotheses have been proposed, such as inhibition of phospholipase, upregulated phospholipid synthesis, increased uptake of phospholipids, and inhibition of intracellular phospholipid traffic. Among them, two hypotheses for the mechanism were tested here: i) excessive cellular uptake of phospholipids and ii) neutralization of intralysosomal pH by drug accumulation. **Methods:** First, RAW264 cells were treated with inhibitors of phospholipid uptake pathways, scavenger receptor-A, LOX receptor, CD36, and pinocytosis, as well as phospholipidosis-inducing drugs. The phospholipid content in the cells were monitored by fluorescence from DiI lipophilic tracer using a flow cytometer. Second, liposomes with an acidic water phase were suspended in drug-containing solutions at neutral pH, and the accumulation of the drug into liposomes was quantitatively measured by high performance liquid chromatography/mass spectrometry. Additionally, the pH of inner phase of the liposomes were measured using fluorescent probe Lysosensor green DND-189. **Results:** Phospholipid content in the cells increased by treatment of phospholipidosis-inducing drugs. Increased cellular phospholipid accumulation did not decrease following treatment with the pathway inhibitors. Regardless of their phospholipidosis-inducing potential, drugs characterized by a high pKa value effectively accumulated in liposomes. This accumulation caused an upward shift of the interior pH of liposomes, which was independent of phospholipidosis-inducing potential of drugs. **Conclusions :** The results suggest that cellular phospholipid-accumulation deos not likely depend on increased uptake via these pathways, nor inhibition of phospholipid transport due to neutralization of acidic organelles.

Keywords-

Drug-induced phospholipidosis, cationic amphiphilic drug, phospholipid uptake, liposomal pH, drug loading

Introduction

Long-term treatment with cationic amphiphilic drugs (CADs) causes hyperaccumulation of phospholipid in cells termed as drug-induced phospholipidosis [1]. Typical tissues affected are from the lung, liver, brain, kidney, skin, and cornea [2]. Lamellar bodies, meaning phospholipid aggregates, are formed in cells, especially in phagocytic cells such as macrophages and Kupffer cells, or alveolar type II cells. Although this type of toxicity was reported years ago [3], the mechanism remains to be elucidated. Phospholipidosis is induced by a wide range of CADs, whereas the symptoms recover by cessation of the drug [4]. Elucidation of this mechanism will lead to safe use of drugs and symptomatic treatments.

Several hypotheses have been proposed as mechanisms of phospholipidosis, including inhibition of phospholipase [5, 6], upregulated phospholipid synthesis [7, 8], increased uptake of phospholipids, and inhibition of intracellular phospholipid traffic [9]. Inhibition of phospholipase classes by CADs has been observed so far, therefore, this is the most widely accepted hypothesis of phospholipidosis. Deterioration of intracellular phospholipid trafficking, which is caused by CAD accumulation in acidic organelles leading to an upward shift in pH, could also be a possible mechanism. In contrast, the other two hypotheses have not been supported by sufficient data. We recently reported that the phospholipidosis-inducing potential of drugs could be rapidly estimated by phospholipase inhibition, binding of drugs to phospholipids, metabolic stability of drugs, and their physicochemical parameters [10]. However, neither uptake of phospholipids nor neutralization of acidic organelle were investigated in that study.

Phospholipids in cells are supplied from

extracellular source of "ready-made" phospholipid as well as intracellular biosynthesis. Low density lipoprotein (LDL) and high density lipoprotein (HDL) are major phoshpolipid carriers in plasma, and supplier of phospholipids. LDL binds to LDL receptor on the cells to be incorporated by endocytosis. Scavenger receptors, responsible for modified LDL incorporation, are also possible pathway for uptake of phospholipid by macrophages. HDL, on the other hand, are not internalized into the cells when transferring cholesteryl esters. It has been reported, however, that scavenger receptor BI (SR-BI), known as HDL receptor, transfer lipoprotein associated phospholipids via endocytosis-independent pathway [11]. Although there are kinds of phospholipid pathways, the distribution of them depends on cell types. In the case of macrophages, it has been reported that SR-A and CD36 plays major part of modified LDL uptake and the contribution of other scavenger receptors are limited [12]. In addition, SR-BI is poorly expressed in macrophages, but instead, is highly expressed in tissues active in cholesterol metabolism [13].

In the present study, the effect of phospholipid uptake was studied by treating mouse peritoneal macrophage-derived cell line, RAW264, with inhibitors of LDL receptor and major scavenger receptors, scavenger receptor-A (SR-A) , LOX receptor (LOX) and CD36, as well as a pinocytic inhibitor, cytochalasin D. In addition, neutralization of acidic liposomes (mimicking acidic organelle) by spontaneous accumulation of basic drugs was compared between CADs. Because the drug accumulation is transporter-independent physicochemical process, we investigated relationship between phospholipidosis-inducing potential and drug accumulation in acidic liposomes.

Materials and methods

Reagents

RAW264 cells were obtained from RIKEN BRC (Tsukuba, Japan) and maintained in RPMI-1640 medium supplemented with fetal bovine serum (FBS) and antibiotics. Polyinosinic acid (poly-I), cytochalasin D (cD) and nile red were obtained from Sigma (Osaka, Japan). Sulfo-*N*-succinimidyl oleate (SSO) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Anti-LDL receptor antibody (LDLR-ab)was supplied by R&D Systems (Minneapolis, MN, USA). Vybrant DiI cell-labeling solution (containing a fluorescent probe DiI for staining of phospholipid bilayer/lamella) and a fluorescent pH probe Lysosensor green DND-189 were purchased from Invitrogen (Osaka, Japan). Choresterol/choresteryl ester quantification kit was product of Biovision (San Francisco, CA, USA). Phosphatidylcholine from chicken eggs was obtained from Wako Pure Chemicals (Osaka, Japan).

Inhibition of phospholipid uptake by pathway inhibitors

The Vybrant DiI cell-labeling solution was added to FBS and maintained overnight at 4ºC. RAW264 cells (passage numbers less than 20) were seeded on a normal plastic well plates at 5×10^4 cells/mL. After 24-h incubation, the cells were treated with a inhibitor (poly-I, 10 mg/L [14]; SSO, 0.4 mM [15]; cD, 1 mg/L [16]; or anti-LDL receptor antibody, 0.5 mg/L [17]; and one of the drugs tested (amiodarone, imipramine, propranolol, chloroquine, chlorpromazine, chloramphenicol, disopyramide, cimetidine and bafilomycin A1; 10 μM each) added to fresh medium containing DiI (1 □L/mL medium). After an additional 24-h incubation, the cells were scraped off from the dish with a silicone scraper and washed with phosphate-buffered saline. In the case of sulfo-*N*-succinimidyl oleate, which is an irreversible CD36 binding inhibitor, the drug was administered 30 min after the inhibitor. The anti-LDL receptor antibody was added 12 h later to avoid a loss in titer. Cellular total cholesterol content treated by poly-I, CD36 or anti-LDL was measured using cholesterol/cholesteryl ester quantification kit following the instruction manual. The washed cells were then treated with propidium iodide (PI) to discriminate living

cells from dead cells. DiI fluorescent intensity was monitored between the wavelengths of 564 and 606 nm with excitation at 488 nm, using a FACSCalibur HG flow cytometer (BD Biosciences, Sparks, MD, USA). Dead cells stained with PI were detected at wavelengths longer than 650 nm and were gated out.

Preparation of liposomes

Phosphatidylcholine from egg yolks dissolved in chloroform was placed in a round-bottom flask and evaporated to a thin film. A citrate buffer (pH 4 or 5) was added to the film to obtain multilamellar vesicles. The vesicles were subjected to freeze–thaw cycles and then passed through a 100-nm pore size polycarbonate filter to obtain large unilamellar vesicles (liposomes). This liposomal suspension was dialyzed overnight against citrate buffer (pH 7.0).

Assessment of drug uptake into liposomes

The liposomal suspension (interior pH, 4) was dialyzed against excess volume of drug-containing isotonic citrate buffer (pH 7.0, 100 mM) under a nitrogen atmosphere. The inner or outer phase of the dialysis bag was subjected to liquid–liquid extraction, solvent evaporation, and reconstitution. Then, drug concentrations in the respective phase were determined using high performance liquid chromatography / tandem mass spectrometry (HPLC/MS/MS). The intraliposomal drug concentration was estimated from the excess drug concentration in the inner phase of the dialysis bag divided by the theoretical volume of the liposomal inner phase.

Evaluation of intraliposomal pH

The fluorescent pH probe, Lysosensor green DND-189 (1 μM), was initially encapsulated into liposomes [18] containing 1.0 mM isotonic citrate buffer (pH 5.0). The outer phase of the resultant probe was removed by dialysis against 1.0 mM isotonic citrate buffer (pH 7.0). Then, the drug was added to the outer phase of liposomes, and the fluorescent intensity was measured at 505 nm (with excitation at 443 nm). A calibration line was prepared from a liposomal suspension with varying intraliposomal pHs [19].

Results

The lipophillic fluorescent probe DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), possess a polar fluorescent headgroup as well as two hydrophobic octadecyl chains. The acyl chains anchor polar fluorescent headgroup on phospholipid bilayer. The phospholipid content in the RAW264 cells was monitored by fluorescence emission from DiI. The results were shown in Fig. 1. Increase in DiI fluorescence suggest that phospholipid accumulation occurred in the cells. Phospholipidosis-inducing drugs showed significant increase in the fluorescence, whereas no significance was observed after treatment with other drugs, suggesting that this assay system properly reflected phospholipid content in the cells. Treatment with bafilomycin A1, which is a vacuolar proton pump inhibitor and neutralize acidic organelle, also increased cellular phospholipid content.

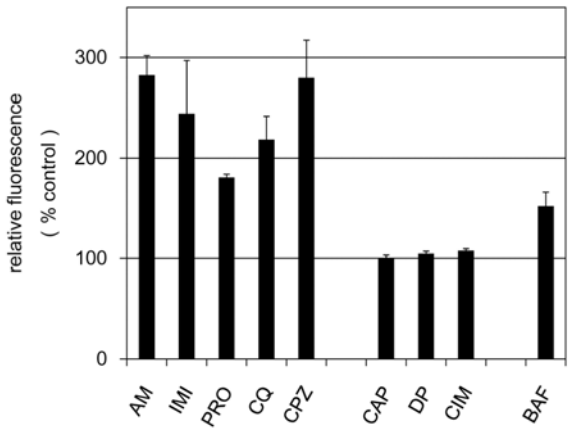

Fig. 1. Drug-induced phospholipid accumulation in RAW264 cells

Cells treated with each drug (10 μM, except for baflromycin A1 at 10 nM) were grown in a medium containing Vybrant DiI lipophilic tracer. Uptake of the fluorescent probe was measured 24 h later using a flow cytometer. The ordinate indicates relative fluorescence ratio between treated and untreated cells with drugs. Asterisk indicates significance ($p < 0.05$, $n = 4$, Tukey's test). AM, amiodarone; IMI, imipramine; PRO, propranolol; CQ, chloroquine; CPZ, chlorpromazine; CAP, chloramphenicol; DP, disopyramide; CIM, cimetidine; and BAF, bafilomycin A1.

Then inhibition study of phospholipid pathway was done using following inhibitors. Poly-I is a polyanionic compound and an inhibitor of SR-A / LOX receptor [14,20], and SSO irreversibly inhibits CD36, a type of oxidized LDL receptor [15]. LDL receptor was also inhibited by anti-mouse LDL receptor antibody. Inhibitorory effect of these inhibitors was confirmed by decrease in cellular total cholesterol content (poly-I: ca. 72%, SSO: ca. 45%, LDLR-ab: ca. 81%, vs. control cells: 100%). Cytochalasin D is a inhibitor of microfilament formation and pinocytosis [16], and therefore, the inhibitorory effect of cD was not easy to be confirmed by cholesterol content. However, the cD was likely to inhibit microfilament formation and pinocytosis because the shape of the cells was spherical under microscope observation. Fig. 2 shows the relative fluorescence of DiI treated cells with/without inhibitors. Treatment with the inhibitors gave no significant shift in DiI cellular fluorescence, suggesting that phospholipid accumulation in the cells was not caused by excessive uptake via these receptors. These results suggest that phospholipid accumulation was not caused by these receptor mediated or receptor nonmediated uptake of LDL.

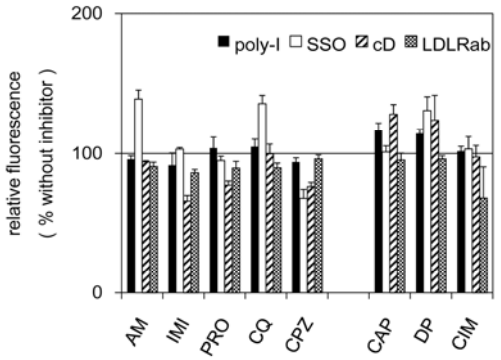

Fig. 2. Effect of pathway inhibitors on cellular phospholipid uptake

The ordinate indicates the relative fluorescence ratio of drug treated cells with / without inhibitors. No significant differences were observed compared to 100% ($p > 0.05$, $n = 3$, Wilcoxon's test). poly-I, polyinosinic acid; SSO, sulfo-*N*-succinimidyl oleate; cD, cytocharasin D; and LDLRab, anti-LDL receptor antibody.

As shown in Fig. 1, treatment by bafilomycin A1 also increased cellular phospholipid content, suggesting that neutralization of acidic organelle leads to phospholipid accumulation. The drugs used in this study except bafilomycin A1 and chloramphenicol were basic, and basic drugs is potent of accumulation into acidic organelle to neutralize them. We hypothesized that basic drugs without phospholipiosis-inducing potential also neutralized the acidic vesicles. If this is true, the phospholipidosis induced by basic drugs is not caused by neutralization of acidic organelle. Then, we investigated drug accumulation in acidic liposomes and phospholipidosis-inducing potential. Figure 3 shows spontaneous drug accumulation in liposomes. Chlorpromazine, a phospholipidosis-inducer, was highly concentrated in artificial vesicles. Other phospholipidosis-inducing drugs (imipramine, propranolol, and chloroquine) also highly accumulated in the vesicles. In contrast, cimetidine and chloramphenicol, which do not induce phospholipidosis, were not concentrated effectively. These results suggest that highly basic drugs spontaneously accumulated in liposomes. However, disopyramide, which is a basic drug without phospholipidosis-inducing potential, showed as much accumulation as the phospholipidosis-inducing drugs. Because the hydrophobicity (cLog*P*) and basicity (p*K*a) of disopyramide are almost equivalent with those of propranolol [10], this result support the hypothesis that loading of drugs into liposomes does not depend on phosphilipidosis-inducing potential.

Then the pH values of the intraliposomal phase were estimated using the fluorescent pH probe Lysosensor Green DND-189 to confirm neutralization of the inner phase by basic drug accumulation. Figure 4 illustrates the effect of liposomes on probe fluorescence (top panel), and fluorescent spectra obtained when using imipramine or disopyramide (bottom panels). The pH-dependent fluorescence of the probe was drastically affected by the presence of a lipid bilayer. Therefore, a calibration line was prepared by dissolving

the pH probe in liposomal suspension. Emission intensity decreased for both drugs after a 1-h incubation of the cells, suggesting neutralization of inner water phase of liposomes by accumulation of the basic drugs. The pH values estimated using the calibration line were 6.2 or higher for both drugs, whereas the initial pH value was adjusted to pH 5.0 (Table 1). Thus, the neutralization effect did not depend on the phospholipidosis-inducing potential of the drugs.

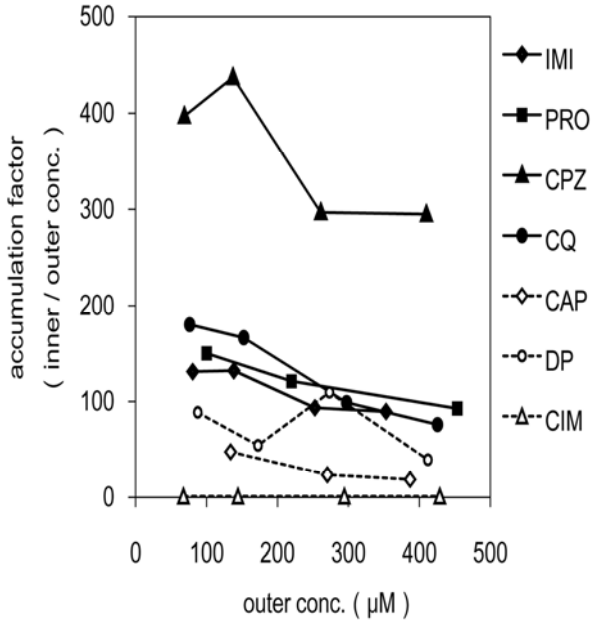

Fig. 3. Spontaneous accumulation of drugs into liposomes containing an acidic inner phase

The liposomal suspension was dialyzed against a drug-containing buffer solution at a neutral pH. After the drug accumulation in liposomes, the drug concentration in the liposomal suspension within the dialysis bag (of known volume) was determined as well as the concentration in the outer solution of the bag. The difference in the concentrations reflected the drug concentration accumulated in liposomes. The theoretical volume of the intraliposomal phase was calculated from the diameter (100 nm) and phospholipid concentration (600 μM), assuming an area of 0.65 nm^2 per phospholipid molecule. Solid and dashed lines indicate phospholipidosis-inducing and non-inducing drugs, respectively. Abbreviations are the same as those in Fig. 1. Only CAP is a neutral drug, whereas the others are cationic. The p*Ka* of CIM is much lower (7.0) than the other basic drugs (> 8.7).

Table 1 Neutralization of the acidic inner phase of liposomes by accumulation of the basic drug

Drug	*Phospholipidosis - inducing Potential*	*Interior pH*		
		Initial	*1 h*	*18h*
Imipramine	+	5.0	6.3	6.2
Disopyramide	-	5.0	6.5	6.2

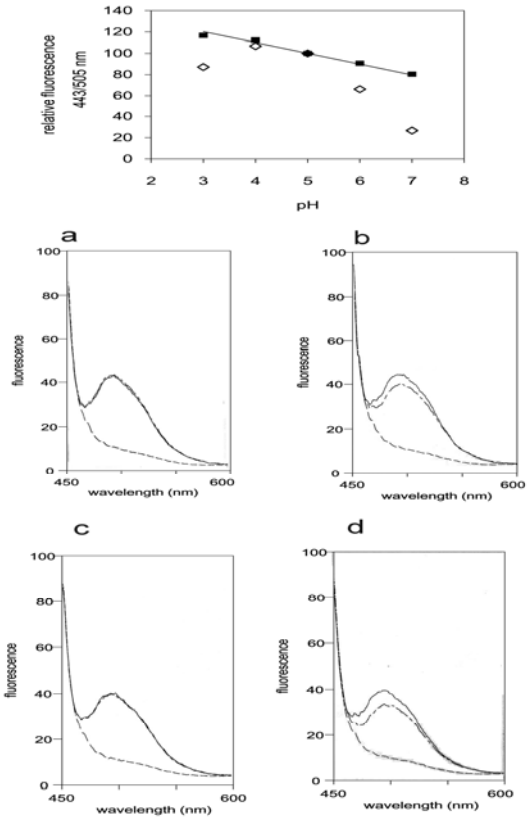

Fig. 4. Neutralization of the acidic intraliposomal phase by basic drug accumulation

Top panel shows the pH dependence of fluorescence emitted from Lysosensor green DND-189 encapsulated in liposomes (black squares). The pH of the extraliposomal buffer was 7.0. The ordinate indicates normalized fluorescence by the intensity at pH 5.0 as a comparison between with and without (white squares) liposomes. Bottom panels show the fluorescence spectra of the probe containing liposomes with the addition of imipramine (a, b) or disopyramide (c, d) in the extraliposomal phase. These spectra were obtained just after adding the drug (a, c) or 1 h later (b, d). The dash-dotted line and the solid line correspond to with and without drug, respectively. The dashed line indicates fluorescence of liposomes alone. Spectra obtained 18 h later were almost the same as those of 1 h later.

Discussion

Long term administration of CADs causes phospholipidosis, and the mechanism of accumulation is still unclear. To date, several hypotheses have been proposed. Phospholipase inhibition is the most widely supported hypothesis. Some studies have reported that phospholipidosis-inducing CADs inhibit classes of phospholipases noncompetitively or competitively, depending on the drug [5, 6]. Upregulated phospholipid synthesis [7,8] is also another potential phospholipidosis mechanism. However, this mechanism is not strongly supported because increased phospholipid synthesis is not observed for a wide range of CADs, and therefore, this may be a specific response of each drug or a type of surrogate response [1]. Increased uptake of phospholipids is another hypothetical mechanism for phospholipid accumulation. Because macrophages excessively take-up oxidized LDL in atherosclerosis, this hypothesis also seems possible. However, supporting data for this hypothesis are not enough. Therefore, we investigated phospholipid uptake in drug-treated RAW264 cells and revealed that phospholipid accumulation was not inhibited by blocking agents of uptake pathways such as the LDL receptor and the major scavenger receptors SR-A, CD36, LOX, as well as an inhibitor of pinocytosis. Although effect of the other minor pathways was not studied here, it is experimentally not easy to confirm contributions of all the pathways, and it is practically reasonable to observe roles of major ones. The results obtained in this study support the hypothesis that increased phospholipid uptake by CADs is not likely responsible for phospholipidosis.

Inhibition of intracellular phospholipid traffic is thought to be another possible cause of phospholipidosis. Although the reason for disrupted traffic is not clearly understood, basic compounds spontaneously accumulate in acidic vesicles [21] and increase the pH. As the loss of the intracellular pH gap disrupts intracellular lipid trafficking [22], a deterioration of the pH gap in cellular compartments can cause lipid transport dysfunction. It was reported that CAD content in lamellar bodies reaches millimolar levels [23]. If such high concentrations of a basic drug are present in a cellular compartment, mathematical estimation of vesicular pH without buffering capacity provides pH 8, when pKa=9 and concentration=1 mM are assumed. In addition, treating cells with bafilomycin A1, a vesicular proton pump inhibitor, increases cell vacuole content [24]. In the present study, bafilomycin A1 also increased DiI fluorescence in the cell, suggesting phospholipid accumulation. From these observations, neutralization of acidic organelles may induce phospholipid accumulation. However, in this study, basic drugs without phospholipidosis-inducing potential also accumulated in acidic liposomes and increased their pHs similar to the phospholipidosis-inducing CADs. If neutralization of cellular acidic compartment caused phospholipidosis, then cationic drugs such as disopyramide would also induce phospholipidosis. For baffilomycin A1, loss in pH gap is a possible cause of dysfunction in lipid sorting and induction of lipid accumulation. However, phospholipidosis induced by wide range of CADs may be caused by other reasons than neutralization of intracellular acidic compartments.

Considering the above observations, increased phospholipid uptake and inhibition of phospholipid traffic are not likely responsible for phospholipidosis. Inhibition of phospholipases, instead, may be the most likely factor responsible for phospholipidosis. Indeed some inconsistencies remain in the past reports, but it seems reasonable to understand that strong binding between CADs and phospholipids noncompetitively inhibits phospholipase activity. Further study will reveal the mechanism of phospholipidosis induction, which will lead to the safe use of drugs.

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