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Effect of Lipid Oxidation on Phospholipidosis - Inducing Drug Binding to Bilayer Membrane

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Abstract
Phospholipidosis-inducing drugs interact with phospholipid and thereby inhibit phospholipases in non-competitive manner. Phospholipids in plasma often suffer from oxidative modification. We studied effect of phospholipid oxidation on the binding affinity of drugs to liposomes. Phospholipid was oxidized by copper sulfate to generate 0.3 % (in mol) 2-thiobarbituric acid reactive substances, which correspond to the oxidative degree of plasma from smokers. Eight drugs with or without phospholipidosis-inducing potential (amiodarone, propranolol, chloroquine, imipramine, chlorpromazine, chloramphenicol, disopyramide, and lidocaine) were subjected to binding displacement study using fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene. Use of these analogous probes enabled us to obtain information about binding depth in the bilayer. Oxidation of phosphatidylcholine increased the affinity of chloroquine binding, while the other drugs were not affected. On the contrary, oxidation of phosphatidylserine did not give any significant effect on cationic drug binding including chloroquine. The results of this study suggest a possibility that phospholipid oxidation enhance the binding affinity of phospholipidosis-inducing drugs.
Keywords: drug-induced phospholipidosis, cationic amphiphilic drug, 1,6-diphenyl-1,3,5-hexatriene (DPH), phospholipid, phosphatidylcholine, phosphatidylserine, oxidation

INTRODUCTION

Drug-induced phospholipidosis is a pathological state characterized by hyperaccumulation of phospholipid in cells[1]. It is caused by long term administration of cationic amphiphilic drugs. Although, mechanism of phospholipidosis has not been understood clearly, several factors affecting have been proposed such as lipophilicity, basicity, and inhibition of enzymatic lipid degradation[1]. The binding between basic drugs and phospholipids has been considered to inhibit lipid degradation enzymes noncompetitively[2]. On the other hand, phospholipid oxidation has been observed under various pathological states such as hyperlipidemia, atherosclerosis, diabetes and smoking[3]. Administration of phospholipidosis–inducing drugs to patients suffering from these symptoms is not a rare case at all, because wide range of drugs possesses both cationic and amphiphilic properties. In this study, effect of phospholipid oxidation on phospholipidosis–inducing drug binding to bilayer membrane was investigated. The depth of drug binding sites and binding parameters (dissociation constants and number of bound drug) were estimated from fluorescence displacement of 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH).

EXPERIMENTAL

Propranolol hydrochloride (PRO), chloroquine diphosphate (CLQ), imipramine hydrochloride (IMI), chlorpromazine hydrochloride (CPZ), chloramphenicol (CAP), disopyramide hydrochloride (DP), lidocaine hydrochloride (LID) were obtained from Wako Pure Chemicals (Osaka, Japan). Amiodarone hydrochloride (AM) was purchased from Sigma (Osaka, Japan). Egg phosphatidylcholine (Egg PC), 1,6-diphenyl-1,3,5-hexatriene (DPH) were product of Wako Pure Chemicals (Osaka, Japan). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was purchased from AnaSpec Inc (San Jose, CA, USA). Synthesized dipalmitoyl phosphatidylserine (DPPS) was purchased from NOF Corporation (Tokyo, Japan). Other reagents were obtained from local suppliers.

Preparation of liposomes and lipid oxidation

Egg PC alone or Egg PC with DPPS (15% DPPS) hydrated in buffer solution was extruded through a polycarbonate filter of 100 nm pore-size. Total phosphorous amount of the prepared unilamellar liposomes was determined by molybdenum blue method. Fleshly prepared vesicles were used in every assay. Lipid oxidation was done by mild incubation of the liposomes with copper (II) sulfate (100 µM), ascorbic acid (500 µM) and hydroperoxide (400 µM) under air atmosphere for 15 minutes at 37℃. Production of thiobarbituric acid reactive substance was quantified from UV absorbance and translated into malondialdehyde amount.

Binding displacement analysis of fluorescent probes by CADs

Binding displacement assay was done following a previous report[4]. Briefly, the fluorescent probe was added to the liposomal suspension. After equilibration under dark, fluorescent emission

intensity (excitation 368 nm, emission 440 nm) of the probe was measured by an RF-540 spectrofluorometer (Simadzu). The dissociation constant of the probe from liposomes was determined by curve-fitting of Langmuir binding isotherm using solver add-in of Microsoft Excel. Binding displacement of DPH by drug was done in similar manner. The dissociation constant of drugs was determined by fitting of a theoretical curve expressing competitive displacement[5]. Without phospholipids, no drugs quenched the fluorescence of the probes in methonol.

RESULTS AND DISCUSSION

It has been reported that phospholipidosis inducing-drug are concentrated in lysosomal lamellar bodies[6]. Therefore in this study, the binding experiments were done under acidic condition (pH 4). It is also known that DPH binds deeply in lipid bilayer to hydrophobic acyl chain of phospholipid, while TMA-DPH locates at intermediate depth interacting with both of acyl group and hydrophilic head group[7]. Therefore, DPH and TMA-DPH can be displaced effectively by drugs binding to deep and shallow sites in the bilayer, respectively. As indicated in Fig.1, among the phospholipidosis-inducing drugs, AM displaced DPH almost completely. CPZ also well competed with both DPH and TMA-DPH. On the other hand, the other phospholipidosis-inducing drugs (IMI, PRO and CLQ) decreased TMA-DPH fluorescence much more than DPH fluorescence. These results suggest that binding sites in the bilayer vary between phospholipidosis-inducing drugs, and that the latter drugs locate at shallower site in the bilayer than CPZ. To date, molecular modelings of drug-phospholipid interactions have been proposed for various drugs including PRO[8], IMI[9] and CPZ[10]. According to these models anticipating that the amino group of drugs interact with phosphate group of phospholipids with the hydrophobic moiety of the drug surrounded by acyl groups of the lipids, the depth of binding sites of these drugs seems quite similar. This discrepancy can be attributed to the stronger hydrophobicity of CPZ than IMI and PRO. (These clog*P*s are 5.2, 4.8 and 3.1, respectively.) CPZ may bind to phospholipid by stronger hydrophobic interaction and displaced DPH more effectively than the two drugs. All of the phospholipidosis-inducing drugs displaced the fluorescent probes, which is consistent with noncompetitive inhibition of phospholipases. However, among drugs without phospholipdosis-indicing potential, CAP (which is a neutral drug) displaced the probes, whereas DP and CIM did not displace the fluorescent probes. These results suggest that phospholipidosis-inducing potential is not explained solely by the binding to phospholipid bilayer, as reported prevoiusly[1]. The dissociation constants of drugs from liposomes were evaluated by regression of a theoretical equation[5] expressing competition between drug and fluorescent plobes (Table 1).

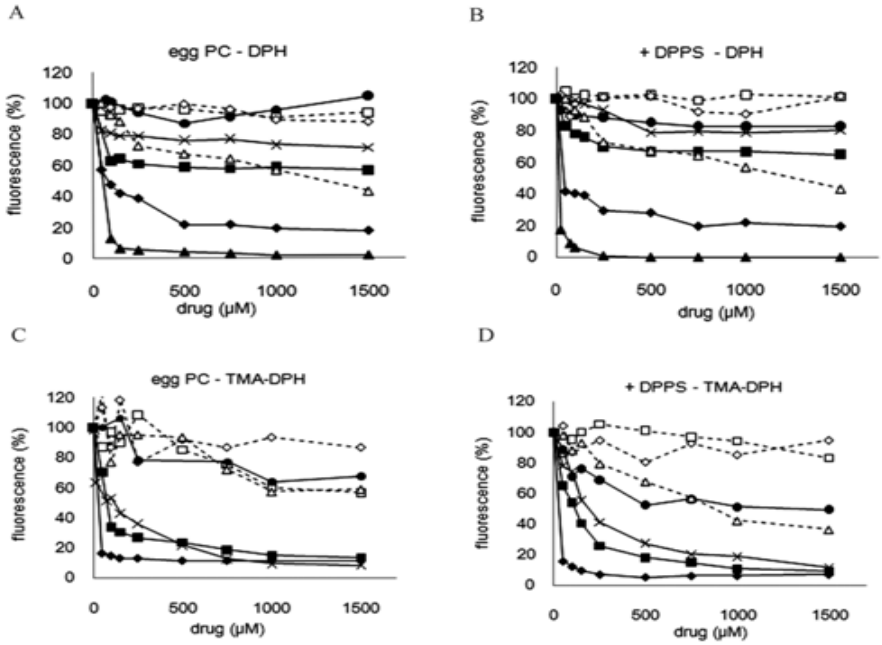

Fig.1 Fluorescence displacement from phospholipid vesicles by drugs at pH 4
Drugs are indicated by following symbols: □, DP; ◊, CIM; Δ, CAP; ●, CLQ; x, PRO; ■, IMI; ♦, CPZ; ▲, AM.

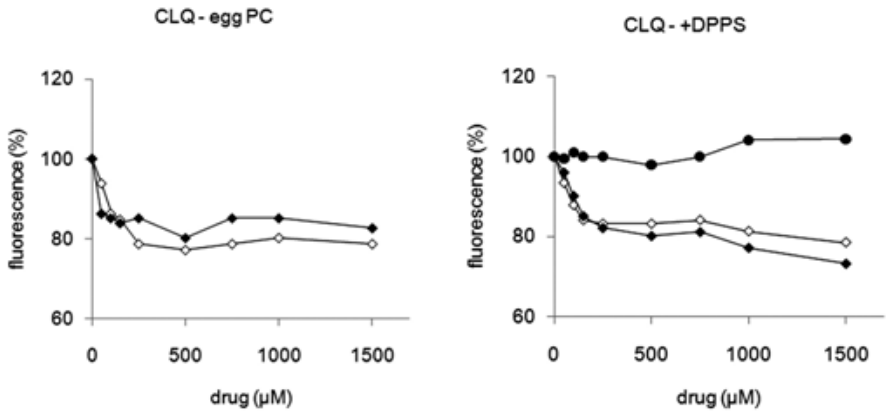

Fig. 2 Effect of lipid oxidation on the fluorescence displacement from phospholipid vesicles by chloroquine

The residual TMA-DPH fluorescence by addition of CLQ was illustrated. Before (◊) and after (♦) phospholipid oxidation. DPH displacement from oxidized liposomes was also traced (●).

Previous paper reported that acidic phospholipids such as phosphatidylserine and phosphatidylgrycerol increased binding of cationic drugs[11]. However, the effect of acidic phospholipids an the drug binding depth in the bilayer is still unclear. The results of the present study (Fig.1) showed that the effect of DPPS (15% mol) was quite limited for many drugs except PRO and CLQ. DPPS increased DPH displacement by PRO, while the fluorescence of TMA-DPH was not affected. Although the reason of this is unknown, previous report[9] put the hypothesis forward that binding depth of PRO shifts by the pH of the water phase. Considering this, DPPS may enhance hydrophilic interaction of PRO with polar head group, resulting in the shift of the binding site to shallower part. For CLQ, addition of DPPS also enabled weak displacement of DPH, suggesting a possibility that the additional negative charge on the carboxy group affects the orientation of CLQ, which lead to interact with hydrophobic acyl chain in the bilayer. This hypothesis is in line with the result that TMA-DPH was more displaced from the DPPS containing liposomes than egg PC liposomes.

In the present study, phospholipid was oxidized by copper sulfate yielding approximately 0.3 % lipid oxidation product (mol malondialdehyde/mol lipid). This oxidation degree corresponds to that in the plasma level after smoking[12]. Lipid oxidation increases electronegative charges on the liposome by production of new carboxyl groups or schiff bases. In order not to deteriorate the newly produced negative charge, we use a neutral buffer (pH 7) for the binding study. Bindings of all drugs except for CLQ were not affected by the lipid oxidation as monitored by DPH displacement. Only for CLQ, TMA-DPH was used to detect the interaction with phospholipids. Fig.2 shows the displacement of the probe by CLQ. By curve fitting, the dissociation constants of the drugs and their numbers of binding were also determined (Table 1).

Table 1 Dissociation constants (*K*) and number of binding site per lipid molecule (*n*)

		***K* (μM)**		***n***	
	Oxidation	−	+	−	+
AM	eggPC	4.1×10^{-7}	1.2×10^{-7}	0.023	0.025
	+ DPPS	5.1×10^{-7}	5.2×10^{-7}	0.021	0.034
CPZ	eggPC	1.5×10^{-6}	5.7×10^{-6}	0.026	0.019
	+ DPPS	3.4×10^{-6}	2.5×10^{-6}	0.033	0.029
PRO	eggPC	4.1×10^{-5}	5.0×10^{-5}	0.025	0.027
	+ DPPS	1.5×10^{-5}	1.3×10^{-5}	0.021	0.027
IMI	eggPC	1.7×10^{-5}	3.1×10^{-5}	0.026	0.027
	+ DPPS	7.0×10^{-5}	3.0×10^{-5}	0.034	0.025
CLQ [1]	eggPC	5.7×10^{-4}	7.3×10^{-5}	0.02	0.042
	+ DPPS	3.8×10^{-4}	2.9×10^{-4}	0.11	0.165
CAP	eggPC	2.9×10^{-4}	2.7×10^{-4}	0.04	0.036
	+ DPPS	1.7×10^{-4}	2.3×10^{-4}	0.029	0.014

[1] determined by TMA-DPH displacement study. Others are obtained from DPH displacement study.

The affinity CLQ to egg PC vesicles were increased by the lipid oxidation. Because CLQ-phospholipid interaction largely depends on electrostatic interaction at polar headgroups of the

phospholipids[13], the negative charges produced by lipid oxidation likely increased the affinity of CLQ to egg PC liposomes. It has also been reported that oxidized acyl group in phospholipid bilayer shift its location upward to surface of the membrane[14]. If this is true, the hydrophobic environment in deep bilayer may not be largely affected by such a moderate oxidation, because oxidized acyl chain is excluded out from the hydrophobic core of the membrane. Therefore, it is not likely that phospholipid oxidation (0.3%) gives large impact on the other phospholipidosis-inducing drugs which interact strongly with the hydrophobic acyl chains. On the contrary, the CLQ affinity to DPPS-containing liposomes was not increased by lipid oxidation, which was inconsistent with the binding to the egg PC liposomes. The reason is still unknown, one of the possible explanations is that increased negative charges produced by oxidative modification increased electrostatic interaction at the surface of the membrane and thereby interfere hydrophobic interaction to acyl chains. No displacement of DPH from oxidized DPPS-containing liposomes (Fig.2) is not conflict of this hypothesis. Clear determination of molecular orientation of oxidized phosphatidylserine will help us to understand this phenomenon.

CONCLUSION

In conclusion, this study suggested that moderate oxidation of phospholipid bilayer enhances CLQ binding to phosphatidylcholine liposomes but does not affect that to phosphatidylserine liposomes. Additionally, the possibility that the depth of PRO binding site shift upward by addition of phosphatidylserine in the bilayer was also suggested.

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