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THE EFFECT OF FERRIPROTOPORPHYRIN IX AND CHLOROQUINE ON PHOSPHOLIPID MONOLAYERS AND THE POSSIBLE IMPLICATIONS TO ANTIMALARIAL ACTIVITY

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Ferriprotoporphyrin IX intercalates into phospholipid membranes, as evidenced from its effect on the surface pressure of monolayers composed of different phospholipids. Ferriprotoporphyrin intercalation is enhanced by membrane hydrophobicity and decreased by negative surface potential. Chloroquine enhances the effect of ferriprotoporphyrin in relatively hydrophobic membranes but reduces it in monolayers composed of highly unsaturated phospholipids. These results are consistent with the differential effect of chloroquine on ferriprotoporphyrin-induced lysis of erythrocytes and of malarial parasites, thus supporting the membrane-lesion hypothesis of antimalarial action.

The mode of action of antimalarial drugs of the aminoquinoline type is not as yet fully understood. Recently, the following mechanism has been suggested. Intraerythrocytic parasites degrade host cell hemoglobin and store the resulting ferriprotoporphyrin IX in the malarial pigment hemozoin (for reviews, see Refs. 1 and 2). The ability to sequester ferriprotoporphyrin is vital to the parasite because free ferriprotoporphyrin can cause osmotic lysis of both free parasite [3,4] and intact erythrocytes [5]. Chloroquine has been shown to bind to ferriprotoporphyrin with very high affinity, yielding a complex which also causes cell lysis. Thus, it has been suggested that the role of chloroquine is to divert ferriprotoporphyrin from the non-toxic malarial pigment into a toxic chloroquine-ferriprotoporphyrin complex [6].

In the course of these investigations, it has been shown that chloroquine enhances the lytic effect of ferriprotoporphyrin in normal mouse erythrocytes [5] but reduces it in the lysis of free parasites [3]. These findings, in conjunction with the demonstrable difference in phospholipid composition of host and parasite (see Ref. 7 for review), could provide a molecular mechanism for the above mentioned differential effects of ferriprotoporphyrin and chloroquine on host and parasite membranes.

In the present work we used the phospholipid monolayer model and we show, from measurements of surface pressure, that ferriprotoporphyrin intercalates between phospholipids, mostly due to hydrophobic interactions, inasmuch as the polar headgroup of the phospholipid has only a marginal effect on ferriprotoporphyrin intercalation. In the presence of chloroquine, ferriprotoporphyrin intercalation is markedly enhanced when the phospholipids constituting the monolayer are relatively saturated. However, as the degree of unsaturation of the acyl chain increases, the effect of chloro-

Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; DPPC, 1,2-dipalmitoyl-sn-phosphatidylcholine, DOPC, dioteoylphosphatidylcholine; DLPC dilinoleylphosphatidylcholine.

quine is reversed, namely, it prevents the expansion of the monolayer by ferriprotoporphyrin. These observations, in conjunction with the relatively higher unsaturation of parasite phospholipids, could constitute the molecular basis for the differential susceptibility of host and parasite membrane to chloroquine in the presence of ferriprotoporphyrin.

Materials were obtained from the following sources: Phosphatidylserine (PS) from bovine brain and cholesterol as well as chloroquine diphosphate and ferriprotoporphyrin IX (equine hemin) were from Sigma. Phosphatidylinositol (PI) from yeast was from Koch-Light. All other chemicals purchased were from the best available grade.

The following phospholipids: 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC), 1,2-dioleoyl-*sn*-phosphatidylcholine (DOPC) and 1,2-dilinoleyl-*sn*-phosphatidylcholine (DLPC), were synthesized according to established procedures [8,9] and purified by high-pressure liquid chromatography [10].

Fresh hemin stock solution (1 mM), was prepared daily by dissolving a weighed aliquot in 0.02 N NaOH at room temperature. After 1.5–2 h, solubilization was completed and the final concentration was adjusted with NaOH after measuring the absorbance at 385 nm ($\epsilon = 6.1 \cdot 10^4$). Thereafter the solution was kept on ice in the dark until use.

Chloroquine was prepared at 1 mM solution in water.

All phospholipids were dissolved at 1 mM in chloroform/methanol (9 : 1, v/v).

Monolayers of the various lipids were formed at the air/water interface in a Teflon trough, and the surface pressure (in $mN \cdot m^{-1}$) was measured by means of a Wilhelmy plate, using a recording microbalance [11]. The initial surface tension (π_i) was recorded, and compounds (ferriprotoporphyrin, chloroquine, etc.) were added to the aqueous subphase (10 mM Tris-HCl/150 mM NaCl (pH 7.4)). The resulting increase in surface tension ($\Delta\pi$) was recorded as a function of time. Further additions of compounds were made only after the surface pressure reached a steady-state level.

Since $\Delta\pi$ depends on π_i and this dependence is characteristic of the lipid as well as the compound added, the appropriate calibration curves were constructed for each lipid or lipid mixture by measuring $\Delta\pi$ vs. π_i as a function of ferriprotoporphyrin concentration. All the results obtained thereafter with chloroquine were normalized to the arbitrary value of $\pi_i = 24$ $mN \cdot m^{-1}$.

The effect of ferriprotoporphyrin on the surface pressure of monolayers made of different lipids is displayed in Table I. It can be seen that the addition of ferriprotoporphyrin to the aqueous subphase causes an increase in the surface pressure, probably due to its intercalation into the monolayer. As the packing of the monolayer is increased by the addition of cholesterol, similar $\Delta\pi$ values are obtained but with significantly lower ferriprotoporphyrin concentrations, as would be expected from the suggested intercalation. Addition of the acidic phospholipids PS or PI reduces the effect of ferriprotoporphyrin on $\Delta\pi$, probably due to negative charge repulsion between the phospholipid head group and the carboxylic groups of hemin, with the consequent decrease in ferriprotoporphyrin dissolution into the monolayer.

TABLE I

EFFECT OF HEMIN AND CHLOROQUINE ON THE SURFACE PRESSURE OF MONOLAYERS MADE OF DIFFERENT LIPIDS

DOPC : PS and DOPC : PI were 1 : 1 (mol/mol) mixtures, and DOPC : cholesterol was 2 : 1 (mol/mol) mixture. All experiments were conducted at room temperature. The results shown are $\Delta\pi$ in $mN \cdot m^{-1}$. The standard deviations of the data were less than 5%.

Lipid: / Additive	DOPC	DOPC : PS	DOPC : PI	DOPC : cholesterol
Chloroquine (1 μM)	0.0	0.0	0.0	0.0
Ferriprotoporphyrin (0.53 μM)	6.9	4.7	4.6	7.9 [a]
Chloroquine (1 μM) → ferriprotoporphyrin	11.9	8.6	1.6	15.3 [a]

[a] [ferriprotoporphyrin] = 0.26 μM.

The addition of chloroquine alone to the subphase has no effect (at 1 μM) on $\Delta\pi$. The subsequent addition of ferriprotoporphyrin considerably increases $\Delta\pi$, in most cases beyond the effect of ferriprotoporphyrin alone. However, while the half-time ($t_{1/2}$) of equilibration of ferriprotoporphyrin alone was in the order of 2–3 mins, in the presence of chloroquine $t_{1/2}$ was greater than 50 mins. Wit DOPC : PI monolayers, the presence of chloroquine reduced the effect of ferriprotoporphyrin on $\Delta\pi$. As the PI used in these experiments was extracted from yeast which has a high content of unsaturated fatty acid [12], the effect of unsaturation on the interactions of ferriprotoporphyrin and chloroquine with monolayers was tested. Results are shown in Table II. As expected, ferriprotoporphyrin had its largest effect on $\Delta\pi$ in DPPC monolayers which are the most densely packed. (Note the lower concentration of ferriprotoporphyrin in this case.) The addition of one double bound and two carbons in the acyl chain (DOPC) produces sufficient amounts of free space for ferriprotoporphyrin intercalation and thus a smaller effect on the surface pressure is observed. The addition of a second double bond (DLPC) has no influence on the effect of ferriprotoporphyrin on $\Delta\pi$.

When ferriprotoporphyrin is added in the presence of chloroquine, its effect on $\Delta\pi$ is enhanced in DPPC and DOPC monolayers but considerably decreased in DLPC monolayers, thus indicating that the degree of unsaturation on the acyl chain dominates under these conditions, similar to the results obtained with DOPC : PI monolayers.

TABLE II

EFFECTS OF HEMIN AND CHLOROQUINE ON THE SURFACE PRESSURE OF MONOLAYERS AS A FUNCTION OF THE DEGRE OF UNSATURATION OF THE CONSTITUENT PC

Results presented are $\Delta\pi$ in $mN \cdot m^{-1}$.

Lipid: Additive	DPPC	DOPC	PLPC
Chloroquine (1 μM)	0.0	0.0	0.0
Ferriprotoporphyrin (0.53 μM)	4.5 [a]	6.9	7.3
Chloroquine (1 μM) → ferriprotoporphyrin	6.8 [a]	11.9	1.4

[a] [ferriprotoporphyrin] = 0.105 μM.

The toxicity of ferriprotoporphyrin and ferriprotoporphyrin-chloroquine complexes to malarial parasite membranes as well as to their host erythrocyte membranes is probably due to lesions formed by these substances and the consequent increase of membrane permeability [13]. Several years ago, Kimelberg and Papahadjopoulos demonstrated that the effectiveness of various soluble proteins in increasing the permeability of phospholipid membranes was directly correlated to their ability to penetrate monomolecular films of the same phospholipid [14]. Therefore, the present work was undertaken with the dual purpose of establishing a molecular basis for the involvement of phospholipids in the alleged lesion formed by ferriprotoporphyrin in biological membranes, and understanding the differential effect of ferriprotoporphyrin and chloroquine on erythrocyte and parasite membranes.

The results presented in this report indicate that ferriprotoporphyrin interacts with membrane interfaces of different lipid compositions. The effect of ferriprotoporphyrin on $\Delta\pi$ in the monolayers of densely packed acyl chains (DOPC : CHOL; DPPC), as compared to the effect on more disordered monolayers (DOPC; DLPC), seems to indicate that ferriprotoporphyrin does indeed intercalate into the monolayer. The effect of acid phospholipid in reducing the effect of ferriprotoporphyrin on surface pressure due to charge repulsion further substantiates this conclusion and suggests that the carboxylic moieties of the terapyrrole ring probably residue outside the phosphate groups. Therefore, in order to carry out its toxic effect on intact cells through penetration and disordering of the lipid domain, ferriprotoporphyrin does not have to reach the inner side of the membrane, as has been previously suggested for the case of erythrocytes [15]. It suffices that ferriprotoporphyrin interdigitates between the phospholipids of the outer monolayer in order to perturb the barrier properties of the lipid bilayer. The presence of cholesterol in the membrane could further enhance the toxic effect of ferriprotoporphyrin. Hence, erythrocyte membranes which contain higher concentrations of cholesterol than parasite membranes [7], are expected be more sensitive to ferriprotoporphyrin, as was indeed found by Fitch and his associates [3,5].

The effect of chloroquine on ferriprotoporphyrin-mediated surface pressure increase is complex. Although chloroquine has been shown to bind to acidic phospholipids [16], it has opposite effects in PS- and PI-containing monolayers; i.e., and increase in the first case and a decrease in the second. Therefore, its action cannot be due exclusively to adsorption to the membrane interface with a resulting change in the surface potential.

The observation that in the presence of chloroquine the half-time of equilibration of the surface pressure, upon addition of ferriprotoporphyrin, is considerably increased as compared to the case in the absence of chloroquine, indicates a complex multi-step process rather than a straightforward modification of the monolayer.

Based on the above mentioned observations, we suggest the following mechanism for the combined effect of ferriprotoporphyrin and chloroquine on the surface pressure of phospholipid monolayers: ferriprotoporphyrin and chloroquine form – in aqueous solution – a multimeric complex [17]. This complex, which has a high association constant [18], can release ferriprotoporphyrin to an appropriate sink, i.e., the phospholipid monolayer. The dissolution (interdigitation) of ferriprotoporphyrin in the monolayer depends on two major factors: the surface potential, which could be reduced by the adsorption of the positively charged chloroquine to the interface of the monolayer, and by the hydrophobic nature of the apolar region. When the acyl chains are more saturated, as well as in the presence of cholesterol, the apolar region becomes more hydrophobic and can extract more ferriprotoporphyrin from the ferriprotoporphyrin-chloroquine complex.

We believe that it is justified to exclude the possibility that the ferriprotoporphyrin-chloroquine complex, as such, interdigitates into the monolayer. If this were so, large aggregates would have had easier access into more loosely packed monolayers; i.e., those composed of unsaturated phospholipids. Evidently, this is not the case. Addition of preformed ferriprotoporphyrin-chloroquine complex at 1 : 1 or 2.5 : 1 concentration ratios to the aqueous phase affected $\Delta\pi$ similarly to the sequential addition of chloroquine and ferriprotoporphyrin.

Taken together, the results presented here are consistent with the observations of Fitch and co-workers that higher ferriprotoporphyrin concentrations are required for the lysis of parasite membranes [3] than those needed for the lysis of their host erythrocytes [5]. The greater hydrophobicity of erythrocyte membranes (based on their larger content of saturated acyl chains of phospholipids and their larger cholesterol content) is also compatible with their higher susceptibility to ferriprotoporphyrin toxicity in the presence of chloroquine. In the parasite membrane (which contains less cholesterol and larger amounts of unsaturated phospholipids) [7], the susceptibility to ferriprotoporphyrin is reduced by the presence of chloroquine.

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