Chapter 6

Effect of Antimalarials Treatment on Rat Liver Mitochondrial lipids

Introduction

Choloroquine(CQ), primaquine(PQ) and quinine(Q) are three major antimalarials used for the treatment of malaria (1). Of the three antimalarials, CQ and PQ accumulate in the acidic vacuoles of the *Plasmodium* and destroy it (1). However, a large proportion of the antimalarials also accumulate in the host tissue cells which harbor the parasite as well as in the non-parasitized cells (2,3). Under experimental conditions, in rat liver lysosomes the concentration of CQ as high as 75 mM has been reported (4). It has also been reported that following treatment with CQ, phospholipids content in the liver increased by about 50 %; the excess phospholipids were found to be localized in lysosomes(5). The ratio of acidic phospholipids to basic phospholipids had increased under this conditions (5). In a recent report Ross et al. have shown that CQ treatment resulted in the induction of cardiolipin synthesis (6). Thabrew et al. have reported that treatment with CQ and Q resulted in alterations in the lipid/ phospholipid composition of the rat liver microsomes (7). Deepalakshmi et al. showed that oral feeding of CQ resulted in increase in the total phospholipids and decrease in cholesterol in rat liver mitochondria (8). It has been suggested that the increased phospholipid content may be attributed to inhibition of various phospholipases by antimalarials (9).

As it was shown in chapter 4 that the three antimalarials CQ, PQ and Q adversely affected the oxidative phosphorylation in rat liver mitochondria as well as the mitochondrial FoF1 ATPase kinetic properties (chapter 5). The process of energy transduction i.e. oxidative phosphorylation is dependent on the membrane phospholipids as is the function of the FoF1 ATPase (10,11). Requirement of acidic phospholipids for FoF1 ATPase is well established (11,12).

While the generalized effects of the antimalarials treatment on liver lipid metabolism have been reported (4,5,7,8), detailed investigations on the effects of treatment with the antimalarials on mitochondrial lipid/phospholipid compositions and membrane fluidity characteristics have not been reported thus far. It was therefore of interest to find out if and in what manner the treatment in vivo with the three antimalarials CQ, PQ and Q affected the mitochondrial lipid/phospholipid profiles. The results of these investigations are summarized in this chapter.

Materials and Methods

Chemicals

1-6 Diphenyl –1,3,5-hexatriene (DPH) and bovine serum albumin fraction V (BSA) were purchased from Sigma Chemical Co. USA. Chloroquine diphosphate, primaquine diphosphate and quinine hydrochloride were purchased from Sigma- Aldrich, USA. Silica Gel G was from E. Merck, Germany. All other chemicals were purchased locally and were of analytical-reagent grade.

The treatment with antimalarials and isolation of mitochondria were essentially the same as described in Chapter 3 and Chapter 4 respectively.

Lipid analysis

Extraction of lipids

Aliquots of mitochondrial suspension containing 4 – 8 mg protein were extracted with 4 ml of freshly prepared chloroform:methanol mixture (2:1 v/v) as described by Folch et al.(13) The tubes were vortexed vigorously allowed to stand at room temperature and the organic phase was carefully removed with the help of a broad gauge syringe. The samples were re-extracted with 3 ml of chloroform:methanol mixture as above and the resulting organic phases were pooled. The pooled chloroform:methanol extracts were treated with 0.1 volume of 0 017% $MgCl_2$, vortexed vigorously, allowed to stand at room temperature and organic phase was carefully removed with care being taken to avoid the proteolipid layer appearing between the organic and aqueous phases. The solvent was completely evaporated under the stream of nitrogen, after which the lipids were dissolved in known volume of chloroform:methanol mixture. Suitable aliquots were taken for the estimation of cholesterol (14) and phospholipid phospohorus in the sample (15) and thin layer chromatography (T.L.C.).

Separation of phospholipids by thin layer chromatography (TLC)

Separation of phospholipid classes was carried out by one dimentional thin layer chromatography (16) using silica gel G A slurry of silica gel G (6 g/13ml distilled water per plate) was prepared by gentle mixing and spread on glass plates with the help of applicator with thickness of layer maintained to 0.25mm. The layer was allowed to dry by leaving plates overnight at room temperature. Prior to use the plates were activated in an oven at 100°C for 20-25 min

Aliquots of the reconstituted samples containing 8-10 µg of phospholipid phosphorus were spotted on TLC plate in a way such that the diameter of the spot was minimum

which ensured better resolution. The conditions for preparation of TLC plates, chamber saturation etc. were according to Stahl (17). The solvent used for the chamber saturation was chloroform : methanol . acetic acid : water (25:15:4:2 v/v). Before run, the plates were reactivated for 2min at 110 ^{0}C. After the run was completed the plates were taken out and kept at room temperature for 3 to 4 hours to remove the solvents.

After brief exposure of iodine vapor spots of individual phospholipid were marked and iodine was allowed to sublime off. After this the spots were carefully scraped and transferred to marked test tubes. To each tube 0.5 ml of 10 N H_2SO_4 were added and the samples were heated on a sand bath for 8-10 hours. The tubes were allowed to cool after which 0 1ml of 70% perchloric acid was added. The tubes were then heated on the sand bath for 3-4 hours till the solution in the tubes were clear and smell of chlorine was undetectable. The analysis of phosphorus content was according to the procedure of Bartlett(15).

Determination of membrane fluidity

Membrane fluidity determination was carried out at 25 °C spectrophotoflourimetrically using DPH as the probe. Stock DPH solution (2mM) was prepared in tetrahydrofuran and stored at 0-4 °C in an amber colored bottle. For measurement of fluorescence polarization, samples were taken in 3ml of buffered sucrose solution (0.25M sucrose containing 10mM Tris-HCl, pH 7.4) at a final protein concentration of 0.2 mg/ml, and an aliquot of stock DPH solution was added so that the molar ratio of probe to lipid was between 1:200 to 1:300 (18,19). The mixture was vortexed vigorously and left in dark for 30 min to permit equilibration of probe into membranes. Fluorescence polarization was

measured in a Shimadzu RF 5000 spectrophotofluorimeter with a polarizer attachment. Excitation and emission wavelengths were 360nm and 430nm; bandwidths were 5nm and 10nm respectively. Data were accumulated for 5 sec for each polarization setting: vertical (parallel) and horizontal (perpendicular) (20). The instrument has program for calculation of fluorescence polarization (P) from which the value of $\bar{r}$, r_α and S can be calculated. The details of the methods have been described previously (20).

Protein estimation was by the method of Lowry et al. with bovine serum albumin used as the standard (21).

Results are given as mean ± SEM.

Statistical evaluation of the data was by Students' t-test.

Results

The results on the effects of antimalarials treatment on the total phospholipid (TPL) and cholesterol (CHL) contents of rat liver mitochondria are summarized in Table 1. As is evident, 7 day treatment with CQ caused a slight increase in the TPL content and decrease in CHL content. However, the changes were not statistically significant. Further corresponding changes in TPL and CHL contents occurred after 14 day treatment with TPL showing a 29 % increase and CHL registering a 19 % decrease. The TPL/CHL molar ratios increased in both the treatment groups progressively with the duration of the treatment.

Table 1. Effect of antimalarials treatment on total phospholipid (TPL), cholesterol (CHL) and TPL/CHL (mole: mole) ratio in rat liver mitochondria.

Treatment	TPL (μg /mg protein)	CHL (μg /mg protein)	TPL/CHL (mole: mole)
Control (16)	163.04±11.80	33.93±2.44	2.33±0.10
CQ-7 (8)	189.71±11.80	31.64±2.04	2.98±0.10 [d]
CQ-14 (8)	209.47±13.11 [b]	27.56±1.36 [a]	3.76±0.10 [d]
PQ-7 (8)	225.32±11.38 [c]	31.40±2.34	3.62±0.10 [d]
PQ-14 (8)	171.85±15.95	25.04±2.76 [a]	3.44±0.14 [d]
Q-7 (8)	182.18±15.81	37.49±2.54	2.44±0.16
Q-14 (8)	183.63±10.80	31.58±2.03	2.92±0.27

The experimental details are as given in the text. The results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p< 0.05$, [b] $p<0.02$, [c] $p<0.002$, and [d] $p<0.001$ compared with the corresponding control.

Following PQ treatment, the TPL content increased only in the initial period, but normalized in 14 day treatment group (Table 1). The effect of PQ treatment on CHL content was the same as that of CQ. As in the case of CQ, the TPL/CHL molar ratio was high for both PQ-7 and PQ-14 day groups.

When Q treatment was given, no changes were seen in both the TPL and CHL contents. The values of TPL were somewhat higher but these were not statistically significant (Table 1).

Further studies were carried to examine the phospholipid composition to find out if the changes in the TPL content could be traced to the alterations in the phospholipid profiles of the mitochondria. From the data in Table 2 it can be seen that CQ treatment caused a drastic decrease (51 to 60 % decrease) in the phosphatidylcholine (PC) component while phosphatidylinositol (PI) component increased by 2.6 and 4.5 folds respectively on 7^{th} and 14^{th} day of treatment. A similar trend (3.1 to 3.6 fold higher values) was also seen for the phosphatidylserine (PS) component. There was a small but reproducible increase in phosphatidylethanolamine (PE) component. Diphosphatidylglycerol (DPG) also increased initially by 66% but in the 14 day group the increase was only about 30%. Sphingomylein (SPM) increased to a 1.4 fold higher value only in the 14 day group.

The magnitude of the changes in phospholipids induced by antimalarials treatment can be appreciated by looking at the contents of the individual phospholipids. These values are given in Table 3 The contents of individual phospholipid components were computed from the TPL content of the individual sample (Table 1) and the % of the said phospholipid in the given sample (Table 2).

Table 2 Effect of antimalarials treatment on phospholipid composition of rat liver mitochondria.

	Phospholipid composition (% of total)						
Phospholipid Class/group	Control (32)	CQ-7 (16)	CQ-14 (16)	PQ-7 (16)	PQ-14 (16)	Q-7 (16)	Q-14 (16)
Lyso	2.67±0.24	3.08±0.73	4.10±0.77	3.17±0.52	4.81±0.28[e]	2.49±0.38	3.45±0.50
SPM	3.75±0.26	4.77±0.69	5.31±0.65[a]	4.16±0.61	6.12±0.41[e]	2.59±0.20[d]	4.09±0.43
PC	43 86±0.46	17.79±2.40[e]	21.48±1.48[e]	37.75±2.10[c]	35.30±0.93[e]	41.40±0.67[c]	42.20±1.88
PI	1.44±0.18	3.78±1.00[a]	6.48±1.21[e]	5.89±0.73[e]	4.45±0.38[e]	1.57±0.40	1.66±0.35
PS	1.46±0.18	5.34±0.55[e]	4.46±0.61[e]	2.89±0.54[b]	4.40±0.30[e]	1.66±0.27	1.07±0.17
PE	30.36±0.34	37.83±1.42[e]	36.51±1.78[d]	30.87±1.38	28.43±0.64[b]	32.14±0.82	30.98±1.25
DPG	16.47±0.49	27.42±1.13[e]	21.65±1.78[c]	15.26±0.56	16.49±0.38	18.16±0.66[a]	16.56±0.72

The experimental details are as given in the text. The results are given as mean ± SEM of the number of observations indicated in the parentheses.
[a] $p< 0.05$, [b] $p<0.02$, [c] $p<0.01$, [d] $p<0.002$ and [e] $p<0.001$ compared with the corresponding control.

Table 3. Effect of antimalarials treatment on phospholipid content of rat liver mitochondria.

	Phospholipid content (μg/mg protein)						
Phospholipid Class/group	Control (32)	CQ-7 (16)	CQ-14 (16)	PQ-7 (16)	PQ-14 (16)	Q-7 (16)	Q-14 (16)
Lyso	4.48±0.48	5.80±1.48	8.77±1.66	7.44±1.43	8.05±0.47 [d]	4.45±0.64	6.51±1.05
SPM	6.34±0.64	9.14±1.51	11.67±1.74 [b]	9.75±1.69	10.30±0.77 [c]	4.58±0.31 [a]	7.63±0.77
PC	71.52±9.68	32.75±3.68 [d]	46.04±4.55 [d]	83.89±4.40	60.96±4.53	75.77±4.54	78.69±3.24
PI	2.47±0.34	7.49±2.18 [d]	13.31±2.25 [d]	13.60±1.94 [d]	7.53±0.47 [d]	2.70±0.65	3.04±0.62
PS	2.36±0.32	10.22±1.26 [d]	9.45±1.37 [d]	6.82±1.48 [b]	7.33±0.47 [d]	3.11±0.62	1.95±0.28
PE	49.69±2.66	72.12±4.77 [d]	75.63±3.67 [d]	69.24±3.56 [d]	49.28±4.02	58.12±1.59 [a]	58.41±3.40
DPG	26.25±1.22	52.19±4.68 [d]	44.60±3.49 [d]	34.58±1.97 [c]	28.42±1.99	33.47±2.72 [c]	31.30±2.01

The experimental details are as given in the text. The results are given as mean ± SEM of the number of observations indicated in the parentheses.
[a] $p< 0.05$, [b] $p<0.01$, [c] $p<0.002$ and [d] $p<0.001$ compared with the corresponding control.

It is clear from the data in Table 3 that CQ treatment resulted in a substantial decrease in the content of PC (36 to 54% decrease) with reciprocal increase (44 to 84 % increase) in SPM content This may perhaps represent a compensatory mechanism. The acidic phospholipids PS and PI showed a substantial 2 to 5 4 fold increase. The mitochondria-specific phospholipid DPG also increased from 70 to 99%. The increase in PE was about 50%.

Changes in the levels of cholesterol and altered phospholipid composition and contents could alter the fluidity of the membrane. This was ascertained by measuring the fluidity of the membranes. It is apparent from the data in Table 4 that CQ-7 group showed a significant increase in the fluidity of the membrane. No change was seen in the 14 day treatment group.

The compositional changes in phospholipids following PQ treatment were similar to those seen after CQ treatment, except that the decrease in PC was somewhat of lesser magnitude (20% decrease) and that the decrease was seen only in the 14 day group. Also, the DPG component was unaffected (Table 2). The quantitative changes are summarized in Table 3 Thus following PQ treatment the contents of lysophospholipids (Lyso) and SPM increased by 66 and 54% respectively in the 7 day group; 14 day treatment showed a further increase (80 and 62% increase respectively). A significant increase of about 4.5 fold was observed in the PI content after 7 day treatment. The 14 day group showed 3 fold increase in the PI content. PS content also increased by 3 fold. The increase in PE and DPG was about 40 and 30% respectively in 7 day group but the values were restored

to the control level in 14 day treatment group (Table 3). However, no change was observed in the mitochondrial membrane fluidity (Table 4).

Quinine treatment showed only marginal changes in the phospholipid composition (Table 2) Thus PC and SPM were reduced (6% and 31% decrease) while a small but reproducible increase was observed in DPG (10% increase) in the 7 day group (Table 2); in 14 day group no changes were evident. The data in Table 3 summarize the quantitative changes. Thus as can be noted, Q treatment caused increase in DPG and PE contents (28 and 17% increases respectively) while the SPM content decreased by 30%. However, no appreciable changes were observed following Q treatment for 14 days. Membrane fluidity was also unaffected (Table 4).

Discussion

From the data presented (Tables1-3), it is clear that treatment with the three antimalarials affected the mitochondria lipid/ phospholipid compositions in different manner. Thus the effects were maximum in CQ treated animals followed by those receiving PQ treatment. Q treatment did not appreciably affect the mitochondrial lipid profiles.

The effects of the three antimalarials on overall lipid/phospholipid profiles in lysosomes, microsomes, mitochondria and erythrocyte membranes have been described (3,4,7,8). Matsuzawa and Hostetler showed that upon treatment with 100mg CQ/ day in diet for 7 consecutive days, the content of TPL increased by 50% (5). Similarly these authors have also shown that the PI content of lysosomes had increased significantly (5). With similar drug regimen Deepalakshmi et al. have shown that the content of TPL in mitochondria

Table 4. The effect of antimalarials treatment on fluidity parameters of rat liver mitochondria.

Treatment Group	Fluidity parameter			
	Fluorescence polarization, p	Fluorescence anisotropy, r	Limited hindered anisotropy, rα	Order parameter, S
Control (38)	0.157±0.003	0.110±0.003	0.047±0.003	0.338±0.012
CQ-7 (24)	0.143±0.004[a]	0.100±0.003[a]	0.034±0.004[a]	0.277±0.020[a]
CQ-14 (24)	0.156±0.004	0.110±0.004	0.046±0.006	0.326±0.021
PQ-7 (24)	0.152±0.004	0.107±0.003	0.042±0.006	0.317±0.018
PQ-14 (24)	0.149±0.005	0.105±0.003	0.039±0.004	0.304±0.018
Q-7 (24)	0.155±0.006	0.109±0.004	0.045±0 005	0.322±0.022
Q-14 (24)	0.157±0.005	0.110±0.004	0.047±0.005	0.331±0.021

The experimental details are as given in the text. The results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p < 0\ 002$ compared with the corresponding control.

increased (58% increase) while the CHL content decreased by 33% (8). The changes reported by these authors (8) are similar to those which we observe here. However, the magnitude of the changes was higher in the studies of Deepalakshmi et al. (8) which could be attributed to the higher dose of CQ employed in their studies (8). The increase in the DPG content following CQ treatment (Table 2 and 3) is consistent with the report of Ross et al.(6).

It has been reported that treatment with Q blocked the synthesis of PC and PE in Jurkat cell lines since Q inhibits the uptake of choline and ethanolamine via the transporter located on the membranes of these cells (22). However, in our studies no effect of Q was observed except for a marginal decrease in PC in 7day group. Thabrew et al. reported alterations in the lipid compositions of the rat liver microsomes following treatment with CQ, PQ and Q (7), using the doses and duration of treatment similar to the regimen we have used. These authors reported that CQ and Q treatments caused decrease in the TPL content and increase in CHL content in the rat liver microsomes (7). The authors further reported an increase in the level of acidic phospholipids PI+PS as well as a decrease in the PC content (7).

All the phospholipid classes except for SPM and DPG are known to be synthesized on endoplasmic reticulum (23.24) The letter two are synthesized by the plasma membrane and mitochondria respectively (23,24). The changes in the microsomal lipids can thus reflect on the alterations in the phospholipid composition of other organelle membrane. Thus the decrease in PC and the increase in PI and PS match and mimic the changes in the microsomal phospholipid changes as reported by Thabrew et al (7). Increased SPM values (Table 2 and 3) suggest that the SPM synthesis in the plasma membrane might

have increased since the microsomal SPM content was also reported to increase following treatment with these drugs (7).

Interestingly, despite the significant changes in the phospholipids the membrane fluidity properties were not altered except in the case of CQ-7 group where the membranes were more fluidized

The membrane fluidity is determined by several parameters, which include mole: mole ratios of TPL/CHL, SPM/PC, SPM/PE and PC/PE (20,25). In order to seek an explanation as to why the fluidity properties were not influenced despite lipid changes, the data was analyzed in terms of molar ratios of specific lipid classes (25,26). These parameters are shown in Table 5. We used additional parameters of molar ratios of acidic phospholipid/ basic phospholipid (APL/ BPL) as suggested by Hostetler et al.(5). These values are also included in the Table. Consistent with the reports of Hostetler et al. the molar ratio of APL/ BPL increased after CQ treatment (5).

We then tried to correlate the p (fluorescence polarization) value with ratios amongst the groups by regression analysis. However, no clear-cut correlationship was emerging. It is possible that the diverse changes in the ratios (Table 5) may have counterbalanced the effects in such a manner that no apparent changes in the fluidity are discernable.

In conclusion our results show that the prolonged treatment with antimalarials can cause significant changes in membrane lipid/ phospholipid composition and thereby results in membrane functions alterations even in the host tissue. Thus the results caution against prolonged treatment with antimalarials by way of prophylaxis.

Table 5 Effect of antimalarials treatment on phospholipid parameters of rat liver mitochondria.

Group	PS+PI	PC/PE	SPM/PC	SPM/PE	TPL/PS	TPL/PI	TPL/PS+PI	APL/BPL	PI/BPL	PS/BPL	DPG/BPL
Control(32)	2.89±0.30	1.45±0.02	0.125±0.009	0.086±0.006	93.26±10.47	121.86±14.92	47.33±5.51	0.250±0.009	0.019±0.003	0.019±0.003	0.213±0.007
CQ-7 (16)	9.12±1.31[e]	0 49±0.08[e]	0.128±0.018	0.350±0.109[b]	20.20±2.06[e]	51 57±15.15[c]	12.72±1.81[e]	0.612±0.039[e]	0.065±0 018[b]	0.090±0.011[e]	0.458±0.025[e]
CQ-14 (16)	10.95±1.62[e]	0.61±0.05[e]	0.159±0.022	0.252±0.037[e]	31.25±5.26[e]	24.84±4.93[e]	13.03±2.42[e]	0.531±0.041[e]	0.108±0.023[e]	0.073±0.011[e]	0.351±0.036[a]
PQ-7 (16)	8.79±1.09[e]	1.29±0.14	0.143±0.022	0.127±0.027	43.91±5.32[e]	21.35±2.69[e]	14.21±1.66[e]	0.338±0.026[c]	0.084±0 012[e]	0 042±0.009[c]	0.212±0.010
PQ-14 (16)	8.85±0.54[e]	1.25±0.04[e]	0.220±0.018[e]	0.179±0.016[e]	24.48±1.84[e]	24.72±1.86[e]	11.94±0.72[e]	0.366±0.016[e]	0.065±0.006[e]	0.064±0.005[e]	0.238±0.008[a]
Q-7 (16)	3.22±0.53	1.30±0.03[d]	0.082±0.008[e]	0.064±0.006[b]	52.51±4.99[e]	74.36±8.52[c]	37.81±4.48	0.284±0.015	0.021±0.006	0.022±0.004	0.241±0.012
Q-14 (16)	2.72±0.44	1.40±0.09	0.135±0.017	0.103±0.014	101.77±14.69	59.87±9.37	43.46±4.48	0.251±0.010	0.022±0.005	0.014±0.002	0.216±0.011

The experimental details are as given in the text. The results are given as mean ± SEM of the number of observations indicated in the parentheses.

[a] $p< 0.05$, [b] $p<0.02$, [c] $p<0.01$, [d] $p<0.002$ and [e] $p<0.001$ compared with the corresponding control.

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Summary

The effect of antimalarials (CQ, PQ and Q) treatment on lipid profiles in rat liver mitochondria were studied. CQ and PQ treatment resulted in an increase in the total phospholipids and decrease in cholesterol levels. The content of acidic phospholipids also increased while the PC content decreased significantly following CQ treatment. Maximum changes were noted with CQ treatment; Q treatment was least effective. CQ treatment also caused an increase in DPG content. The molar ratio of lipid/ phospholipid changed significantly. However, despite these changes in the phospholipid profiles the mitochondrial membrane fluidity was not altered to an appreciable extent. The results suggest that long- term treatment with antimalarials alter the lipid parameters.