Chapter 5

Effect of Picrotoxin-induced Epileptic Condition and Antiepileptic Drug Treatment on Rat Brain Mitochondrial Lipids

---

Introduction

Intense neuronal activity during seizure episodes may cause structural and functional changes in plasma and subcellular membranes. Neuropathological investigations have repeatedly pointed seizure related alterations of neurons characterized by swollen and often disrupted mitochondria (1). Brain damage during seizure episodes is related to increased intracellular Ca^{2+} levels $[Ca^{2+}]_i$, which has an important role in the regulation of diverse spectrum of cellular events as well as in epileptogenesis (2). Additionally, intense seizure activity is reported to be associated with inhibition of microsomal Mg^{2+},Ca^{2+}-ATPase mediated Ca^{2+} uptake and deregulation of Ca^{2+} homeostasis (3, 4). Induction and progression of seizures and maintenance of essential electrolyte balance are critically dependent on the membrane function.

Repeated seizure episodes lead to membrane degradation and accumulation of bioactive lipids (5). Increased proteolytic inactivation of brain mitochondrial monoamine oxidases is associated with endogenous stimulation of lipid peroxidation in the epileptic condition (6). Lipid peroxidation induced mitochondrial dysfunction and reduced energy levels have been reported by several researchers, suggesting that neuronal injuries are caused by excessive generation of ROS (7-9). Treatment with AEDs is reported to be associated with modulating mitochondrial and microsomal membrane bound enzymes and function (10-12). Valproate treatment caused mitochondrial cytopathy, inhibition of mitochondrial

fatty acid oxidation and formation of the abnormal mitochondrial structure that could directly affect lipid metabolism in muscle. (13).

As it was shown in chapter 2 and 3 that chronic epileptic condition and treatment with AEDs adversely affected oxidative energy metabolism as well as the ATPase kinetic properties in rat brain mitochondria. Mitochondrial respiratory chain and F_oF_1ATPase are membrane bound moieties and proficient functioning of this system are crucially dependent on membrane lipid/phospholipid composition. Therefore, it was of interest to carry out detailed investigations on the effects of PTX-induced epileptic condition and AED treatment on mitochondrial lipid/phospholipid compositions and membrane fluidity characteristics. The results of these investigations are summarized in this chapter.

Materials and methods

Chemicals

1,6-diphenyl-1,3,5 hexatriene (DPH), bovine serum albumin (BSA) and PTX were purchased from Sigma Chemical Co. (St. Louis, USA). Slica gel G was from E. Merck, Germany. All other chemicals were purchased locally and were of analytical reagent grade.

The treatment with PTX and AEDs were as shown in Chapter 2 and 3. The animals were killed by decapitation after the end of treatment period (i.e. on the day 8th after AEDs treatment and day 21st after PTX or PTX-AEDs treatment). Isolation of mitochondria was essentially the same as described in Chapter 2.

Lipid analysis

Extraction of lipids

Aliquots of mitochondrial suspension containing 4 to 8 mg protein were extracted with 4 ml freshly prepared chloroform:methanol (2:1)mixture as described by Folch *et al.* (14). 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 sample 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 0.2 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 evoparated 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 (15) and phospholipid phosphorous in the sample (16) and thin layer chromatography (TLC).

Separation of phospholipids by thin layer chromatography (TLC)

Separation of phospholipid classes was carried out by one dimensional thin layer chromatography (17) using Silica gel G. A slurry of silica gel G (6g/13 ml distilled water per plate) was prepared by gentle mixing and spread on glass plates with the help of de Saga applicator with thick ness of layer maintained to 0.25 mm. 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.

Aliquotes of reconstituted samples containing 8-10 µg of phospholipid phosphorous were spotted on TLC plate in a way such that the diameter of the spot was minimum which ensured better resolution. The conditions of preparation of TLC plates, chamber saturation etc was according to Stahl (18). The solvent used for the chamber saturation was chloroform : methanol : acetic acid : water (25 : 15 : 4 : 2 v/v). The chamber was saturated for at least 2 to 3 hrs. Before run, the plates were reactivated for about 1 min at 110 °C. After the run was completed the plates were taken out and kept at room temperature for 3 to 4 hours to remove solvents.

After brief exposure of iodine vapor, spots of individual phospholipid were marked and inodine 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 sampled were digested on a sand bath (300-400 °C) for 8-10 hrs. The tubes were allowed to cool after which 0.1 ml of 70% perchloric acid was added. The tubes were then heated on the sand bath for 3-4 hrs to ensure complete digestion and till the solution in the tubes

were clear and smell of chlorine was undetectable. The analysis of phosphorous content was according to the procedure of Katewa and Katyare (16).

Determination of membrane fluidity

The measurements on membrane fluidity was carried out with 1,6-diphenyl-1,3,5 hexatriene (DPH) as the probe following the procedures described earlier in details (19, 20). Briefly, DPH stock 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 3 ml of buffered sucrose solution (0.25 M sucrose containing 100mM Tris-HCl buffer, pH 7.4) at a final protein concentration of 0.2 mg/ml, and an aliquot of stock DPH solution was added such that the molar ratio of probe to lipid was between 1:200 to 1:300 (20, 21). The mixture was vortexed vigorously and left in dark for 30 min to permit equilibration of probe into membranes. Fluorescence polarization (P) was measured at 25 °C in a Shimadzu RF-5000 spectrophotofluorimeter with a polarizer attachment. Excitation and emission wavelengths were 360 nm and 430 nm; bandwidths were 5 nm and 10 nm respectively. Data were accumulated for 5 sec for each polarization setting: vertical (parallel) and horizontal (perpendicular) (19). The instrument has an inbuilt program for calculating and printing out fluorescence polarization (P) values. Calculations for fluorescence anisotropy (r), limited hindered anisotropy (r_∞) of the fluorophore and order parameter (S) was done as described by van Blitterswijk *et al.* (21).

Protein estimation was done by the method of Lowry *et al.* (1951) with BSA as the standard (22).

Results are given as mean ± SEM. Statistical evaluation of the data was performed using the Students' *t*-test.

Results and Discussion

The results on the effects of PTX treatment on the total phospholipid (TPL) and cholesterol (CHL) content of rat brain mitochondria are summarized in Table 1. As is evident that TPL and CHL content decreased significantly by 15% and 17% respectively without affecting the TPL / CHL molar ratio.

Further studies were carried out to examine the phospholipid composition to find out if the changes in the TPL content be traced to the alterations in the phospholipid profiles of the mitochondria. From the date given in Table 2 it can be seen that in PTX-induced epileptic condition lysophospholipid (lyso), sphingomyelin (SPM) and phosohatidylcholine (PC) components increased by 1.3 to 2 fold; while phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) were reduced by 17 to 78%, with maximum lowering was observed for DPG (Table 2).

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

As can be noted from the data in Table 3 that content of acidic phospholipids (PS, PI and DPG) was reduced after PTX treatment by 37 to 81% with maximum lowering in the DPG content (81% decrease).

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 by using DPH as the probe. It is apparent from the data given in Table 1 that brain mitochondrial membrane was somewhat more fluidized under the epileptic condition.

CBZ treatment to the control animals caused 40 to 46% lowering of TPL and TPL/CHL molar ratio (Table 4). Lyso, SPM and PS components were elevated by 1.2 to 1.6 folds, whereas PI, PC and DPG components were reduced by 16 to 30% (Table 7). LTG treatment on other hand decreased CHL content by 16% (Table 5) with increased SPM, PS and PE components and decreased PC and DPG components (12-24% decrease) as shown in Table 9. CBZ and LTG treatment to the control animals lead to increased

Table 1. Effect of PTX-induced seizures on total phospholipid and cholesterol content, and membrane fluidity of brain mitochondria

	Parameter	Control	PTX
Total phospholipid	TPL (μg/mg protein)	555.43 ± 7.970 (8)	470.11 ± 15.46 (8)***
Cholesterol	CHL (μg/mg protein)	512.85 ± 19.01 (8)	427.04 ± 11.94 (8)**
TPL / CHL	Molar ratio	0.54 ± 0.022 (8)	0.55 ± 0.017 (8)
Membrane fluidity	Fluorescence polarization (P)	0.202 ± 0.0041 (16)	0.183 ± 0.0038 (12)*
	Fluorescence anisotropy (r)	0.145 ± 0.0032 (16)	0.130 ± 0.0029 (12)*
	Limited hindered anisotropy (r∝)	0.093 ± 0.0042 (16)	0.073 ± 0.0039 (12)**
	Order parameter (S)	0.483 ± 0.0121 (16)	0.429 ± 0.0141 (12)**

The results are given as mean ± SEM of the number of observation indicated in the parentheses.

*, $p<0.01$; **, $p<0.002$; ***, $p<0.001$.

Table 2. Effect of PTX-induced convulsions on phospholipid COMPOSITION of brain mitochondria

Phospholipid class	Phospholipid composition (% of total)		Change (%)
	Control	PTX	
Lyso	5.15 ± 0.31	6.93 ± 0.26***	+35
SPM	5.13 ± 0.39	10.31 ± 0.62***	+101
PC	32.85 ± 1.51	43.75 ± 0.66***	+33
PS	3.41 ± 0.28	2.56 ± 0.25*	-25
PI	4.10 ± 0.25	2.61 ± 0.14**	-36
PE	37.99 ± 0.95	31.59 ± 0.69***	-17
DPG	11.21 ± 0.30	2.51 ± 0.25***	-78

The results are given as mean ± SEM of 8 independent observations.
Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
*, $p<0.05$; **, $p<0.02$; ***, $p<0.001$

Table 3. Effect of PTX-induced convulsions on phospholipid CONTENT of brain mitochondria

Phospholipid class	Phospholipid content (μg/mg protein)		Change (%)
	Control	PTX	
Lyso	25.62 ± 1.81	32.48 ± 1.31	-
SPM	26.18 ± 1.10	48.08 ± 2.20*	+84
PC	180.72 ± 9.52	205.83 ± 7.50	-
PS	18.82 ± 0.28	11.92 ± 1.10*	-37
PI	23.32 ± 0.25	12.24 ± 0.65*	-48
PE	212.7 ± 5.06	148.97 ± 6.92*	-30
DPG	62.33 ± 2.17	11.79 ± 1.24*	-81

The results are given as mean ± SEM of 8 independent observations.
Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
*,$p<0.001$

222

Table 4. Effect of Carbamazepine (CBZ) treatment on total phospholipid and cholesterol content, membrane fluidity of brain mitochondria in control and epileptic condition.

Parameter	Control	CBZ	PTX treated	PTX-CBZ
Total phospholipid (μg/mg protein)	540.6 ± 20.44 (8)	289.8 ± 17.88 (8)**	445.1 ± 8.63**	262.5 ± 14.75**, a
Cholesterol (μg/mg protein)	498.2 ± 18.10 (8)	445.9 ± 17.99 (8)	383.3 ± 12.90**	285.7 ± 14.82**, a
TPL / CHL (Molar ratio)	0.54 ± 0.023 (8)	0.33 ± 0.023 (8)**	0.58 ± 0.009	0.47 ± 0.025*, a
Membrane fluidity:				
Fluorescence polarization (P)	0.212 ± 0.0049 (16)	0.185 ± 0.0038 (24)**	0.181 ± 0.0035 (16)**	0.301 ± 0.0012 (16)**, a
Fluorescence anisotropy (r)	0.152 ± 0.0037 (16)	0.132 ± 0.0029 (24)**	0.129 ± 0.0026 (16)**	0.223 ± 0.0010 (16)**, a
Limited hindered anisotropy (r∝)	0.103 ± 0.0050 (16)	0.075 ± 0.0038 (24)**	0.078 ± 0.0050 (16)*	0.197 ± 0.0014 (16)**, a
Order parameter (S)	0.508 ± 0.0130 (16)	0.434 ± 0.0110 (24)**	0.441 ± 0.0150 (16)*	0.707 ± 0.0025 (16)**, a

The results are given as mean ± SEM of the number of observation indicated in the parentheses.
*, $p<0.01$ and **, $p<0.001$ compared with Control group; a, $p<0.001$ compared with PTX treated group.

Table 5. Effect of Lamotrigine (LTG) treatment on total phospholipid and cholesterol content, membrane fluidity of brain mitochondria in control and epileptic condition.

Parameter	Control	LTG	PTX treated	PTX-LTG
Total phospholipid (μg/mg protein)	556.6 ± 16.04 (8)	503.2 ± 22.11 (8)	450.2 ± 9.02***	403.0 ± 16.8$^{***, a}$
Cholesterol (μg/mg protein)	499.5 ± 13.77 (8)	421.7 ± 16.62 (8)**	401.6 ± 10.04***	363.8 ± 15.30$^{***, a}$
TPL / CHL (Molar ratio)	0.57 ± 0.017 (8)	0.59 ± 0.012 (8)	0.56 ± 0.013	0.71 ± 0.050$^{***, a}$
Membrane fluidity:				
Fluorescence polarization (P)	0.197 ± 0.0075 (16)	0.199 ± 0.0028 (24)*	0.180 ± 0.0040 (16)**	0.311 ± 0.0029 (16)$^{***, b}$
Fluorescence anisotropy (r)	0.140 ± 0.0058 (16)	0.142 ± 0.0022 (24)*	0.130 ± 0.0030 (16)	0.228 ± 0.0024 (16)$^{***, b}$
Limited hindered anisotropy (r∝)	0.083 ± 0.0052 (16)	0.090 ± 0.0030 (24)*	0.076 ± 0.0047 (16)	0.207 ± 0.0031 (16)$^{***, b}$
Order parameter (S)	0.463 ± 0.0210 (16)	0.475 ± 0.0081 (24)*	0.434 ± 0.0140 (16)	0.716 ± 0.0059 (16)$^{***, b}$

The results are given as mean ± SEM of the number of observation indicated in the parentheses.
PTX-LTG treated: PTX-induced epileptic animals treated with LTG
*, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$ compared with Control ; a, $p<0.05$ and b, $p<0.001$ compared with PTX treated group.

Table 6. Effect of Clobazam (CLB) treatment on total phospholipid and cholesterol content, membrane fluidity of brain mitochondria in control and epileptic condition.

Parameter	Control	CLB	PTX treated	PTX-CLB
Total phospholipid (μg/mg protein)	534.1 ± 9.49 (8)	496.1 ± 14.10 (8)*	454.9 ± 7.44***	438.9 ± 14.77***
Cholesterol (μg/mg protein)	519.6 ± 12.50 (8)	464.9 ± 25.86 (8)	419.6 ± 6.01***	416.2 ± 22.27***
TPL / CHL (Molar ratio)	0.51 ± 0.011 (8)	0.54 ± 0.034 (8)	0.57 ± 0.012**	0.54 ± 0.033
Membrane fluidity:				
Fluorescence polarization (P)	0.204 ± 0.0050 (16)	0.244 ± 0.0120 (24)**	0.182 ± 0.0043 (16)**	0.277 ± 0.0058 (16)***, a
Fluorescence anisotropy (r)	0.146 ± 0.0040 (16)	0.178 ± 0.0094 (24)**	0.134 ± 0.0033 (16)**	0.203 ± 0.0047 (16)***, a
Limited hindered anisotropy (r∞)	0.096 ± 0.0045 (16)	0.137 ± 0.0130 (24)**	0.073 ± 0.0044 (16)**	0.171 ± 0.0063 (16)***, a
Order parameter (S)	0.485 ± 0.0150 (16)	0.579 ± 0.0028 (24)**	0.426 ± 0.0130 (16)**	0.657 ± 0.0120 (16)***, a

The results are given as mean ± SEM of the number of observation indicated in the parentheses.
*, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$ compared with Control; a, $p<0.001$ compared with PTX treated group.

Table 7. Effect of Carbamazepine (CBZ) treatment on brain mitochondrial phospholipid COMPOSITION in control and epileptic condition

Parameter	Control	CBZ	PTX treated	PTX-CBZ treated
Lyso	5.27 ± 0.22	8.53 ± 0.21***	6.29 ± 0.23**	9.30 ± 0.45***, a
SPM	6.04 ± 0.09	8.59 ± 0.28***	9.72 ± 0.28***	11.41 ± 0.25***, a
PC	31.11 ± 0.34	33.81 ± 0.62	40.32 ± 0.49***	20.81 ± 0.26***, a
PS	5.01 ± 0.20	4.36 ± 0.23*	3.91 ± 0.22**	9.60 ± 0.22***, a
PI	5.18 ± 0.11	3.62 ± 0.08***	3.97 ± 0.27***	6.29 ± 0.41*, a
PE	35.72 ± 0.42	30.10 ± 0.80***	33.62 ± 1.05	34.71 ± 0.41
DPG	11.7 ± 0.32	8.77 ± 0.21***	2.43 ± 0.04***	7.80 ± 0.39***, a

Phospholipid composition is given as % of the Total phospholipids. The results are given as mean ± SEM of 8 independent observations. Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-CBZ treated: PTX-induced epileptic animals treated with CBZ
*, $p<0.02$; **, $p<0.01$ and ***, $p<0.001$ compared with Control group
a, $p<0..001$ compared with PTX treated group.

Table 8. Effect of Carbamazepine (CBZ) treatment on brain mitochondrial phospholipid CONTENT in control and epileptic condition

Parameter	Control	CBZ	PTX treated	PTX-CBZ treated
Lyso	28.41 ± 1.32	24.96 ± 2.13	28.13 ± 1.15	24.36 ± 1.17^{*,a}
SPM	32.65 ± 1.26	29.78 ± 1.85	43.22 ± 1.30**	29.95 ± 0.99^{b}
PC	176.4 ± 6.46	100.56 ± 5.47**	179.4 ± 0.49	52.96 ± 1.24^{**,b}
PS	18.57 ± 1.38	12.69 ± 1.13**	17.43 ± 1.06	25.23 ± 0.87^{**,b}
PI	28.0 ± 1.27	10.58 ± 0.84**	17.70 ± 1.34**	16.53 ± 1.13**
PE	193.2 ± 8.13	87.51 ± 6.44**	149.4 ± 5.31**	91.11 ± 1.79^{**,b}
DPG	63.23 ± 3.14	24.84 ± 1.66**	9.92 ± 0.77**	20.42 ± 0.91^{**,b}

Phospholipid content is given as μg of phospholipid per mg protein The results are given as mean ± SEM of 8 independent observations.
Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-CBZ treated: PTX-induced epileptic animals treated with CBZ
*, $p<0.05$ and **, $p<0.001$ compared with Control group; a, $p<0.05$ and b, $p<0.001$ compared with PTX treated group.

Table 9. Effect of Lamotrigine (LTG) treatment on brain mitochondrial phospholipid COMPOSITION in control and epileptic condition

Parameter	Control	LTG	PTX treated	PTX-LTG treated
Lyso	4.89 ± 0.13	5.20 ± 0.37	6.26 ± 0.19***	4.80 ± 0.28 b
SPM	5.34 ± 0.17	7.34 ± 0.69*	9.83 ± 0.27***	5.81 ± 0.20 b
PC	35.43 ± 0.34	31.33 ± 0.60***	40.27 ± 0.50***	34.81 ± 1.19 b
PS	3.86 ± 0.20	4.38 ± 0.15**	3.70 ± 0.17	4.19 ± 0.24
PI	4.78 ± 0.11	4.39 ± 0.12	3.58 ± 0.13***	3.93 ± 0.29
PE	36.05 ± 0.66	39.50 ± 0.45***	34.04 ± 0.95	41.93 ± 1.25***, b
DPG	9.95 ± 0.14	7.53 ± 0.14***	2.32 ± 0.013***	4.52 ± 0.38***, a

Phospholipid composition is given as % of the Total phospholipids. The results are given as mean ± SEM of 8 independent observations. Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-LTG treated: PTX-induced epileptic animals treated with LTG
*, $p<0.02$; **, $p<0.01$ and ***, $p<0.001$ compared with Control group
a, $p<0.05$ and b, $p<0.001$ compared with PTX treated group.

Table 10. Effect of Lamotrigine (LTG) treatment on brain mitochondrial phospholipid CONTENT in control and epileptic condition

Parameter	Control	LTG	PTX treated	PTX-LTG treated
Lyso	27.69 ± 0.76	26.40 ± 2.75	28.17 ± 1.41	19.34 ± 1.21$^{***, a}$
SPM	31.38 ± 1.05	36.98 ± 1.46**	44.24 ± 1.59***	23.48 ± 1.12$^{***, a}$
PC	202.9 ± 3.81	157.6 ± 7.28***	181.2 ± 7.25*	140.24 ± 5.14$^{***, a}$
PS	19.74 ± 1.56	21.96 ± 0.99	16.65 ± 0.99	16.99 ± 1.24
PI	23.14 ± 1.25	22.05 ± 1.05	16.11 ± 1.34**	15.95 ± 1.39**
PE	204.4 ± 5.10	198.8 ± 9.49	153.2 ± 6.41***	168.6 ± 5.27***
DPG	56.38 ± 1.89	37.82 ± 1.54***	10.44 ± 1.02***	18.12 ± 1.37$^{***, a}$

Phospholipid composition is given as % of the Total phospholipids. The results are given as mean ± SEM of 8 independent observations. Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-LTG treated: PTX-induced epileptic animals treated with LTG
*, $p<0.02$; **, $p<0.002$ and ***, $p<0.001$ compared with Control group
a, $p<0.001$ compared with PTX treated group.

Table 11. Effect of Clobazam (CLB) treatment on brain mitochondrial phospholipid COMPOSITION in control and epileptic condition

Parameter	Control	CLB	PTX treated	PTX-CLB treated
Lyso	4.30 ± 0.10	4.12 ± 0.18	6.22 ± 0.15**	6.22 ± 0.19**
SPM	5.04 ± 0.22	10.44 ± 0.29**	9.94 ± 0.25**	12.50 ± 0.32**, a
PC	36.56 ± 2.68	34.29 ± 0.78	40.23 ± 0.49	28.01 ± 0.49*, a
PS	4.29 ± 0.19	3.90 ± 0.19	3.48 ± 0.13*	3.76 ± 0.15*
PI	4.70 ± 0.20	2.54 ± 0.17**	3.50 ± 0.27*	3.40 ± 0.16**
PE	34.98 ± 2.50	40.57 ± 1.42	34.50 ± 0.83	34.07 ± 0.67
DPG	9.79 ± 0.21	4.17 ± 0.34**	2.22 ± 0.031**	12.14 ± 0.40**, a

Phospholipid composition is given as % of the Total phospholipids. The results are given as mean ± SEM of 8 independent observations. Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-CLB treated: PTX-induced epileptic animals treated with CLB.
*, $p<0.01$; **, $p<0.001$ compared with Control group
a, $p<0.001$ compared with PTX treated group.

Table 12. Effect of Clobazam (CLB) treatment on brain mitochondrial phospholipid CONTENT in control and epileptic condition

Parameter	Control	CLB	PTX treated	PTX-CLB treated
Lyso	22.92 ± 0.78	20.52 ± 1.27	29.71 ± 1.45***	27.30 ± 1.01*
SPM	36.37 ± 1.43	51.68 ± 1.75***	47.36 ± 2.00***	54.88 ± 1.63***, a
PC	195.2 ± 3.34	170.2 ± 5.86*	192.0 ± 8.22	122.93 ± 2.50***, b
PS	23.01 ± 1.29	19.39 ± 1.24	15.64 ± 0.98**	14.48 ± 0.69***
PI	25.19 ± 1.38	12.52 ± 0.76***	16.73 ± 1.52***	14.90 ± 0.73***
PE	186.9 ± 2.89	201.3 ± 5.97*	167.7 ± 7.84*	149.4 ± 2.92***
DPG	52.30 ± 1.67	20.52 ± 1.54***	10.40 ± 1.23***	53.31 ± 1.90 b

Phospholipid composition is given as % of the Total phospholipids. The results are given as mean ± SEM of 8 independent observations. Lysophospholipid (lyso), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG).
PTX-CLB treated: PTX-induced epileptic animals treated with CLB
*, $p<0.05$; **, $p<0.002$ and ***, $p<0.001$ compared with Control group
a, $p<0.02$ and b, $p<0.001$ compared with PTX treated group.

Table 13. Effect of PTX treatment on phospholipids parameters of brain mitochondria

Parameter	Control	PTX
PS+PI	7.55 ± 0.54	5.21 ± 0.392**
PC/PE	0.88 ± 0.06	1.39 ± 0.039***
SPM/PC	0.16 ± 0.01	0.236 ± 0.028*
SPM/PE	0.14 ± 0.01	0.330 ± 0.028***
TPL/PS	172 ± 16.8	200.6 ± 25.20
TPL/PI	139 ± 9.36	184 ± 12.63**
TPL/PS+PI	73.6 ± 3.15	90.25 ± 4.877**
APL/BPL	0.25 ± 0.007	0.090 ± 0.005***
PI/BPL	0.054 ± 0.003	0.030 ± 0.0031**
PS/BPL	0.150 ± 0.044	0.029 ± 0.0030***
DPG/BPL	0.543 ± 0.021	0.548 ± 0.0171

The results are given as mean ± SEM of 8 independent observations.
Shingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), Acidic phospholipids (APL), Basic phospholipids (BPL); *, $p<0.05$ **, $p<0.02$ and ***, $p<0.001$

Table 14. Effect of Carbamazepine (CBZ) treatment on phospholipids parameters of brain mitochondria in control and epileptic condition

Parameter	Control	CBZ	PTX treated	PTX-CBZ
PS+PI	6.69 ± 0.275	7.99 ± 0.245	7.89 ± 0.490	15.91 ± 0.77*,
PC/PE	0.92 ± 0.015	1.17 ± 0.047*	1.20 ± 0.026*	0.581 ± 0.010^{*,a}
SPM/PC	0.19 ± 0.003	0.296 ± 0.010*	0.244 ± 0.005*	0.566 ± 0.017^{*,b}
SPM/PE	0.17 ± 0.003	0.345 ± 0.019*	0.291 ± 0.009*	0.329 ± 0.008^{*,b}
TPL/PS	158 ± 11.40	67.66 ± 5.401*	114 ± 4.35*	27.41 ± 0.62^{*,b}
TPL/PI	105 ± 4.36	79.75 ± 3.92*	112 ± 4.93	42.76 ± 2.29^{*,b}
TPL/PS+PI	62.6 ± 3.05	36.37 ± 2.15*	56.44 ± 2.32	16.51 ± 0.54^{*,b}
APL/BPL	0.27 ± 0.009	0.223 ± 0.007*	0.124 ± 0.004*	0.358 ± 0.0091^{*,b}
PI/BPL	0.070 ± 0.002	0.048 ± 0.001*	0.048 ± 0.002*	0.095 ± 0.0061^{*,b}
PS/BPL	0.047 ± 0.003	0.058 ± 0.004*	0.047 ± 0.002	0.145 ± 0.0034^{*,b}
DPG/BPL	0.157 ± 0.005	0.117 ± 0.003*	0.030 ± 0.001*	0.118 ± 0.0063^{*,b}

The results are given as mean ± SEM of 8 independent observations.
Shingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), Acidic phospholipids (APL), Basic phospholipids (BPL). *, $p<0.001$ compared with Control group; a, $p<0.001$ compared with PTX treated group

Table 15. Effect of Lamotrigine (LTG) treatment on phospholipids parameters of brain mitochondria in control and epileptic condition

Parameter	Control	LTG	PTX treated	PTX-LTG
PS+PI	7.56 ± 0.47	8.77 ± 0.130*	7.30 ± 0.438	8.131 ± 0.536
PC/PE	0.99 ± 0.04	0.80 ± 0.023***	1.19 ± 0.024***	0.841 ± 0.054*
SPM/PC	0.16 ± 0.005	0.236 ± 0.006***	0.245 ± 0.006***	0.168 ± 0.008 ^{b}
SPM/PE	0.15 ± 0.006	0.187 ± 0.003***	0.289 ± 0.008***	0.140 ± 0.008 ^{b}
TPL/PS	168 ± 9.252	116 ± 8.061***	121 ± 4.08***	97.85 ± 4.14***
TPL/PI	142 ± 5.70	115 ± 6.29**	125 ± 5.85	105 ± 6.44***
TPL/PS+PI	73.6 ± 3.34	57.65 ± 3.19**	61.65 ± 2.25**	49.57 ± 1.81$^{***, a}$
APL/BPL	0.23 ± 0.004	0.209 ± 0.003**	0.115 ± 0.004***	0.15 ± 0.007$^{***, b}$
PI/BPL	0.053 ± 0.003	0.056 ± 0.002	0.043 ± 0.002*	0.048 ± 0.004
PS/BPL	0.045 ± 0.003	0.056 ± 0.002**	0.044 ± 0.002	0.051±0.003$^{***, b}$
DPG/BPL	0.129 ± 0.002	0.096 ± 0.002 ***	0.028 ± 0.001***	0.055±0.005$^{***, a}$

The results are given as mean ± SEM of 8 independent observations.
Shingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), Acidic phospholipids (APL), Basic phospholipids (BPL);
*, $p<0.05$ **, $p<0.01$ and ***, $p<0.001$ compared with Control; a, $p<0.05$ and b, $p<0.001$ compared with PTX treated group

Table 16: Effect of Clobazam (CLB) treatment on phospholipids parameters of brain mitochondria in control and epileptic condition

Parameter	Control	CLB	PTX treated	PTX-CLB
PS+PI	9.10 ± 0.395	6.43 ± 0.32**	7.01 ± 0.399*	7.20 ± 0.313**
PC/PE	1.02 ± 0.073	0.85 ± 0.010*	1.17 ± 0.040	0.83 ± 0.026*, b
SPM/PC	0.14 ± 0.008	0.31 ± 0.010**	0.247 ± 0.006**	0.45 ± 0.015**, b
SPM/PE	0.14 ± 0.008	0.26 ± 0.009**	0.290 ± 0.015**	0.369 ± 0.014**, b
TPL/PS	125 ± 3.855	129 ± 6.25	132 ± 6.27	118 ± 5.24 b
TPL/PI	114 ± 3.436	202 ± 15.13**	136 ± 11.91	131 ± 6.88***
TPL/PS+PI	58.7 ± 1.79	78.4 ± 4.41**	64.90 ± 2.38**	60.9 ± 1.73
APL/BPL	0.24 ± 0.012	0.13 ± 0.005**	0.109 ± 0.005**	0.259 ± 0.005 b
PI/BPL	0.060 ± 0.003	0.03 ± 0.002**	0.041 ± 0.0034**	0.046 ± 0.002 *
PS/BPL	0.054 ± 0.003	0.046 ± 0.002*	0.041 ± 0.0017**	0.050 ± 0.002 a
DPG/BPL	0.124 ± 0.005	0.049 ± 0.002**	0.026 ± 0.0040**	0.163 ± 0.006**, b

The results are given as mean ± SEM of 8 independent observations. Shingomyelin (SPM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), Acidic phospholipids (APL), Basic phospholipids (BPL);

*, $p<0.05$ and **,$p<0.001$ compared with Control; a, $p<0.05$ and b, $p<0.001$ compared with PTX treated group

membrane fluidity of brain mitochondria (Table 4 and 5). CLB treatment to the control animals showed marginal effects on TPL and CHL contents (Table 6) while PT and DPG component decreased by 46 to 57% (Table 11). Membrane fluidity was decreased after CLB treatment to the control animals (Table 6). The individual phospholipid contents (µg/mg of protein) in general were found to be decreased in all the AEDs treatment to control animals as shown in Table 8, 10 and 12.

CBZ treatment to the epileptic animals resulted into 1.2 to 1.9 folds increase in Lyso, SPM, PS and PI components; while DPG and PC were decreased significantly as compared to controls (Table 7). Drastic decrease (43 to 51%) in the TPL and CHL contents was seen with composite decrease in the TPL/CHL ratio (Table 4). While LTG treatment to the epileptic animals imparts restoration of Lyso, SPM, PC, PI and PS to the normality with slight increase in PE while DPG was decreased by 55% (Table 9). When the epileptic animals were treated with CLB, decreased PS, PI and PC was observed with 1.2 to 1.5 fold elevation in Lyso and DPG (Table 11). Both, LTG and CLB treatment to the epileptic animals decreased the TPL and CHL contents by 18-27% with increased mitochondrial membrane rigidity (Table 5 and 6). Similar trend was observed in individual phospholipids content in treatment with AEDs to the epileptic animals (Table 8, 10 and 12).

In general, AEDs treatment to the control non-epileptic animals caused drastic alterations with respect to disproportionate decrease in the content of individual phospholipids.

Differential alterations are observed in TPL and CHL contents and membrane fluidity parameters. When epileptic animals were treated with AEDs, TPL and CHL contents were reduced as compared to controls. Interestingly decreased membrane fluidity was the common finding in all the AED treatment epileptic animals.

Requirement of phospholipids for the optimum functioning of mitochondrial membrane proteins is well documented (23). Results on the mitochondrial membrane properties indicated drastic alterations in membrane composition and molar ratios of phospholipids parameters. ATPase activity is known to be dependent on acidic phospholipids, in particular PS and PI. Hence, observed changes in the kinetic parameters of ATPase (Chapter 3) could be attributed to altered phospholipid composition and distorted membrane charge distribution. Also, mitochondrial membrane became more fluidized under the epileptic condition (Table 1). Interestingly, AEDs treatment to the epileptic animals was showed increase rigidity of the membrane beyond control values (Table 4-6). Additionally, treatment with AEDs to the control animals showed differential effects on fluidity. The membrane fluidity is determined by several parameters, which include mole:mole ratios of TPL/CHL, SPM/PC, SPM/PE, PC/PE etc (19, 24).

In order to seek and explanation as to why the fluidity properties were influenced after either PTX or AEDs treatment, the data were analyzed in terms of molar ratios of specific lipid classes (24, 25). These parameters are shown in Table 13-16. To summarize, PTX-induced convulsions resulted in to decrease in almost all the parameters (Table 13)

whereas LTG and CLB treatment showed a general reduction in these parameters (Table 14, 15). Opposite was true for CBZ treatment with either control or epileptic animals (Table 16). It is also possible that substantial increase in lysophospholipid content - that is evident in either PTX-epileptic or AEDs treated groups - may lead to the fluidization of the membrane, since lysophosphoglycerides can exert detergent like action on the membranes (26). Increased lyosophospholipids indicate activation of phospholipases, which are known to be activated by Ca^{2+} (27). Mitochondrial function is crucial to intracellular Ca^{2+} homeostasis and possesses several Ca^{2+} transport system (28). The concentration of free Ca^{2+} is coupled to neuronal membrane potential and in turn has been shown to be critically dependent on the functioning of mitochondria (29). Activation of excitatory neurotransmitter receptor leads to Ca^{2+} influx into the neurons and increasing free $[Ca^{2+}]_i$ (30) which has been implicated in the pathophysiology of epilepsy (31). However, chronic seizure activity could lead to modulation of aforementioned systems and thereby it could activate the Ca^{2+} dependent downstream pathways.

Decreased PI (in epileptic animals and AED treated control animals) might be related to membrane degradation, as PI hydrolysis is often a feature of stimulated phospholipid turnover (32). This observation is supported by earlier reports that decreased PI could also be associated with seizure-induced excessive release of excitatory neurotransmitters and activation of phospholipases (PLA_2 and PLC) mediated signaling pathway (5). Arachidonate is released from *sn2* position of glycerol backbone, which is implicated in

neuronal plasticity, ischemic brain damage and epilepsy (33). Brain levels of free fatty acids and DAG rise rapidly with onset of seizures, reflecting activation of PLA_2 and PLC (34). To conclude, massive alteration in the distribution of the individual phospholipids of the brain mitochondrial membrane as observed in the epileptic condition could lead to dysfunction of important respiratory chain components. However, AEDs treatment was failed to restore these variations in the epileptic animals.

Reference

1. Meldrum, B.S. Excitotoxicity and selective neuronal loss in epilepsy. Brain Pathol. (1993) 3, 405-412.
2. MacDonald, J.W., Garofalo, E.A., Hood, T., Sackellares, J.C., Gliman, S., McKeever, P.C., Troncoso, J.C., Johnston, M.W. Altered excitatory and inhibitory amino acid receptor binding in hippocampus of patients with temporal lobe epilepsy, Ann. Neurol. 29 (1991) 529-541.
3. Parsons, J.T., Churn, S.B., DeLorenzo, R.J. Chronic inhibition of cortex microsomal $Mg^{2+}Ca^{2+}$ ATPase-mediated Ca^{2+} uptake in the rat pilocarpine model following epileptogenesis, J. Neurochem. 79 (2001) 319-327.
4. Costa, M.S., Funke, M.G., de Meis, L., Cavalheiro, E.A., da G. Naffah-Mazzacoratti, M. Status epilepticus induced by pilocarpine and Ca^{2+} transport by microsome in the hippocampus of rats, Neurosci. Lett. 366 (2004) 292-296.
5. Bazan, N.G., Tu, B., Rodriguez de Turco, E.B. What synaptic lipid signaling tell us about seizure-induced damage and epileptogenesis. Prog Brain Res. 135 (2002) 175-185.
6. Medvedev, A.E., Gorkin, V.Z. Endogenous stimulation of lipid peroxidation in brain increases proteolytic inactivation of mitochondrial monoamine oxidases. Int J Dev Neurosci (1994)12, 151-5.
7. Cock, H.R. The role of mitochondria and oxidative stress in neuronal damage after brief and prolonged seizures. Prog. Brain Res. (2002) 135, 187-196.

8. Gupta, R.C., Milatovic, D., Dettbarn, W.D. Nitric oxide modulates high-energy phosphates in brain regions of rats intoxicated with diisopropyl-phosphofluoridate or carbofuran by N-tert-butyl-α-phenylnitrone or vitamin E. Arch. Toxicol, (2001) 75, 346-356.

9. Ueda, Y., Yokoyama, H., Niwa, R. *et al,* Generation of lipid radicals in the hippocampal extracellular space during kainic acid induced seizures in rats. Epilepsy Res. (1997) 26, 329-333.

10. Ponchaut, S., van Hoof, F., Veitch, K. Cytochrome aa3 depletion is the cause of the deficient mitochondrial respiration induced by chronic valproate administration. Biochem Pharmacol (1992) 43, 644-647.

11. Veitch. K, Hue, L. Flunarizine and cinnarizine inhibit mitochondrial complexes I and II: possible implication for parkinsonism. Mol Pharmacol (1994) 45, 158-163

12. Tong, V., Teng, X.W., Chang, T.K., Abbott, F.S. Valproic acid II: effects on oxidative stress, mitochondrial membrane potential, and cytotoxicity in glutathione-depleted rat hepatocytes. Toxicol Sci. (2005) 86, 436-443.

13. Melegh, B., Trombitas, K. Valproate treatment induces lipid globule accumulation with ultrastructural abnormalities of mitochondria in skeletal muscle. Neuropediatrics (1997) 28, 257-261.

14. Folch, J., Less, M., Sloane-Stanley, G.H. A simple method for isolation and purification of total phospholipids from animal tissues. J. Biol. Chem. (1957) 226, 497-509.

15. Zlatkis, A., Zak, B., Boyle, J.A. A new method for determination of serum cholesterol. J. Lab. Clin. Med. (1953) 41, 486-492.

16. Katewa, S.D. and Katyare, S.S. A simplified method for inorganic phosphate determination and its application for phosphate analysis in enzyme assays. Anal. Biochem. (2003) 323, 180-187.

17. Skipiski, V.P., Barelay, M., Barelay, R.K. *et al.* Lipid composition of human serum lipoprotein. Biochem. J. (1967) 104, 340-361.

18. Stahl E. Apparatus and general techniques. In: Thin Layer Chromatography: A Laboratory Handbook (2^{nd} Ed) Stahl E. (Eds.) Springer-Verlag, New York, 1969, pp 52-86.

19. Bangur, C.S., Howland, J.L., Katyare, S.S. Thyroid hormone treatment alters phospholipid composition and membrane fluidity of the rat brain mitochondria. Biochem. J. 305 (1995) 29-32.

20. Mehta, J.R., Braund, K.G., Hegreberg, G.A., Thukral, V. Lipid fluidity and composition of the erythrocyte membrane from healthy dogs and Labrador retrievers with hereditary muscular dystrophy. Neurochem. Res. 16 (1991) 129-135.

21. van Blitterswijk, W.S., van Hoeven, R.P., van der Meer, B.W. Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady state fluorescence polarization measurements. Biochim. Biophys. Acta 644 (1981) 323-332.

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. Protein measurement with Folin phenol reagent. J. Biol. Chem. (1951)193, 265-275.

23. Daum, G. Lipids of mitochondria. Biochem. Biophys. Acta. (1985) 822, 1-42.

24. Parmar, D.V., Ahmed, G., Khandkar, M.A., Katyare, S.S. Mitochondrial ATPase: a target for paracetamol-induced hepatotoxicity. Eur J Pharmacol (1995) 293, 225-229.

25. Senault, C., Yazbeck, J., Goubern, M., Portet, R., Vincent, M., Gallay, J. Relation between membrane phospholipid composition, fluidity and function in mitochondria of rat brown adipose tissue. Effect of thermal adaptation and essential fatty acid deficiency. Biochim. Biophys. Acta. (1990) 1023 , 283-288.

26. Rossiter, R.J. and Strickland, K.P., 1960. The metabolism and function of phosphatides. In: Bloch, K. (Ed.), *Lipid Metabolism.* p 69.

27. Trimenstein, M.A. and Nelson, S.D. Subcellular binding and effects on calcium homeostasis produced by acetaminophen and non-hepatotoxic regioisomer, 3' hydroxyacetanilide, in mouse liver. J. Biol. Chem. (1989) 264, 1814.

28. Nicholls, D.G. A role for the mitochondrion in the protection of cells against calcium overload? Prog. Brain Res. (1985) 63, 97-106.

29. White, R.J. and Reynolds, I.J. Mitochondria and Na^{+}/Ca^{2+} exchange buffer glutamate-induced calcium loads in cultured cortical neurons. J. Neurosci. (1995) 15, 1318-1328.

30. MacDermott, A., Mayer, G.L., WestBrook, G.L., Smith, S.J., Barker, J.C. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons, Nature (Lond.) 321 (1986) 519-522.

31. Ure, J.A., Perassolo, M. Update on the pathophysiology of the epilepsy, J Neurol. Sci. 177 (2000) 1-17.

32. Procellati, G., Arienti, G. Handbook of Neurochemistry, 2nd Ed. 3 (1983) 133-161.

33. Bazan, N.G. Arachidonic acid in the modulation of excitable membrane function and at the onset of brain damage. Ann N Y Acad Sci. 559 (1989) 1-16.

34. Visioli, F., Rodrigues de Turco, E.B., Kreisman, N.R., Bazan, N.G. Membrane lipid degradation is related to interictal cortical activity in a series of seizures. Metab Brain Dis (1994) 9, 161-170.

Summary

Mitochondrial respiratory chain including F_oF_1ATPase is membrane bound moiety; hence studies were extended to examine the effect of PTX-induced seizures and AEDs treatment on the lipid composition of mitochondria. After PTX treatment TPL and CHL content decreased significantly by 15% and 17% respectively without affecting the TPL / CHL molar ratio. Fluidization of mitochondrial membrane was seen. The content of acidic phospholipids (PS, PI and DPG) was reduced after PTX treatment with maximum lowering in the DPG content, indicating altered charge distribution in the membrane micro-environment. AEDs treatment to the control non-epileptic animals, in general, caused drastic alterations with respect to disproportionate decrease in the content of individual phospholipids. Differential alterations are observed in TPL and CHL contents and membrane fluidity parameters. When epileptic animals were treated with AEDs, TPL and CHL and contents were reduced as compared to controls, individual phospholipids contents showed differential alterations. Decreased membrane fluidity was the common finding in all the AED treated epileptic animals. In conclusion, drastic alteration in the distribution of the individual phospholipids of the brain mitochondrial membrane as observed in the epileptic condition could lead to dysfunction of important respiratory chain components. However, AEDs treatment was failed to restore these variations in the epileptic animals.