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Structural and functional alterations in mitochondrial membrane in picrotoxin-induced epileptic rat brain

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Abstract

Mitochondrial function is a key determinant of both excitability and viability of neurons. Present studies were carried out to decipher cerebral mitochondrial oxidative energy metabolism and membrane function in the chronic condition of generalized seizures induced by picrotoxin (PTX) in rats. PTX-induced convulsions resulted in decreased respiration rates (14–41%) with glutamate, pyruvate + malate, and succinate as substrate. The ADP phosphorylation rates were drastically reduced by 44–65%. An opposite trend was observed with ascorbate + *N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as substrate. In general, uncoupling of the mitochondrial electron transport was observed after PTX treatment. Malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities were decreased by 20–80%; also, there was significant reduction in cytochrome *b* content after PTX treatment, while the F_oF_1 ATPase (complex V) activity increased in basal and 2,4-dinitrophenol (DNP)-stimulated condition, indicating increased membrane fragility. The substrate kinetics analysis had shown that K_m and V_{max} of the higher affinity kinetic component of ATPase increased significantly by 1.2- to 1.4-fold in epileptic condition. Temperature kinetic analysis revealed 1.2-fold increase in energies of activation with decreased transition temperature. The total phospholipid (TPL) and cholesterol (CHL) contents decreased significantly with lowering of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), while lysophospholipid (lyso), sphingomyelin (SPM), and phosphatidylcholine components were found to be elevated. Brain mitochondrial membrane was somewhat more fluidized in epileptic animals. Possible consequences of mitochondrial respiratory chain (MRC) dysfunction are discussed. In conclusion, impairment of MRC function along with structural alterations suggests novel pathophysiological mechanisms important for chronic epileptic condition.

Keywords: Picrotoxin; Epilepsy; Mitochondria; ATPase; Oxidative phosphorylation; Membrane fluidity; Cytochromes; Phospholipids profile

Introduction

Mitochondrial oxidative phosphorylation is the primary source of energy for neuronal metabolism. About 40% of the total ATP is consumed by Na^+K^+-ATPase and Ca^+Mg^+-ATPase, which maintains the neuronal plasma membrane potential and intracellular Ca^{2+} sequestration (Astrup et al., 1981; Herrington et al., 1996; Thayer and Miller, 1990). Mitochondrial function is a crucial determinant of cell death and oxidative stress, thus it acts as the "stress sensor", and in extreme circumstances "executioner" of the cell (Green and Reed, 1988). Additionally, the mitochondrion plays an important role in neurotransmitter metabolism (Waagepetersen et al., 1999). There are accumulated evidences suggesting that epileptic seizures can occur as a presenting sign of mitochondrial dysfunction in the central nervous system (Canfoglia et al., 2001; DiMurro et al., 1999).

Neuronal activity can cause short-term changes of mitochondrial membrane potential that serves as an important factor for regulating mitochondrial permeability transition (MPT) (Kromer and Reed, 2000). Additionally, long-term changes of oxidative phosphorylation resulting from modulation of mitochondrial gene expression in response to increased synaptic activity have been described (Williams et

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al., 1998). Intense seizure activity causes massive influx of Ca^{2+} through voltage-gated and *N*-methyl-D-aspartate (NMDA)-dependent ion channels (Van den Pol et al., 1996), which results in elevated intracellular and intramitochondrial Ca^{2+} thus leading to mitochondrial membrane depolarization, thereby resulting in energy failure and superoxide production (Gupta and Dettbarn, 2003; Liang et al., 2000; Schuchmann et al., 1999). This could trigger the acute neuronal cell death that occurs after status epilepticus (Fujikawa et al., 2000). Alterations in neurotransmitter concentrations in synapse (Jope and Morrisett, 1986; Schwarcz et al., 1978; Walton et al., 1990), reduced plasma membrane Na^+K^+-ATPase activity, cellular pH and residual ATP content in subcellular fractions, and lysosomal dysfunction in rat brain have also been reported in some animal models of epilepsy (Acharya et al., in press; Folbergrova et al., 1985; Kobayashi et al., 1990; Walton et al., 1999). Deficiency of complex I of respiratory chain is documented in patients with temporal-lobe epilepsy (Kunz et al., 2000). In contrast to the short-term alterations of mitochondrial function by epileptoform activity, it remains unclear whether and how exactly cerebral mitochondrial structure–function is altered under the long-term epileptic condition.

Many species of animals do develop spontaneous seizures; however, these cases are sporadic and not usually suitable for experimentation (Fisher, 1989). Several animal models are being used to study epilepsy (Fisher, 1989). This includes use of various chemical epileptogens such as kainic acid, picrotoxin (PTX), pentylenetetrazole, bicuculine, and so forth, electrically kindled and genetic models like photosensitive baboons, DBA/2J, E1, and totterer mice, genetically epilepsy prone rats (GEPRs), and so forth. In the present study, the picrotoxin (PTX)-induced animal model was used to investigate long-term changes in mitochondrial structural and functional properties in epileptic rat brain. Mitochondrial function was characterized by assessing oxidative energy metabolism, kinetic attributes of ATP-synthesizing enzyme F_oF_1ATPase, and activities of dehydrogenases in rat brain mitochondria. Studies were also carried out to evaluate possible membrane alterations in terms of lipid/phospholipid composition and fluidity. The results of these investigations are summarized in the present communication.

Materials and methods

Chemicals

Picrotoxin (PTX), sodium salts of succinic, pyruvic, L-malic, and oxaloacetic acids, ATP, ADP, rotenone, Triton X-100, NADH, dichloroindophenol (DCIP), 1,6-diphenyl-1,3,5 hexatriene (DPH), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). L-glutamic acid was obtained from E. Merck (Dramstadt, Germany). *N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and 2,4-dinitrophenol (DNP) were purchased from British Drug Houses (Dorset, Poole, England). Ascorbic acid was from Sarabhai Chemicals (Vadodara, India). All other chemicals were purchased locally and were of analytical reagent grade.

Animals and treatment with PTX

Male albino rats of Charles–Foster strain (200–250 g) were used. The animals had free access to food and water. PTX solution was prepared fresh daily in saline and was injected intraperitoneally (ip) at the dose of 1.5 mg/kg body weight for 20 consecutive days (Mazarati et al., 1992). Initially, the animals developed seizures within 30 min of PTX administration. Tonic–clonic convulsions were well established within 8–10 days of treatment. At later stages, seizures developed within 10–20 min of PTX administration. The controls were given equivalent volume of saline. There was no mortality of rats.

The animals were kept in individual cages and observed for incidences, character, and intensity of epileptic manifestations. The animals were scored according to the scale described by Kubova et al. (1992) as indicated below:

0 No changes
1 Uneasiness, scratching tremor, single myoclonic jerks
2 Atypical minimal seizures
3 Minimal seizures consisting of clonic convulsions involving the head and forelimb muscles and leaving righting reflexes intact
4 Major seizures without the tonic phase
5 Complete major seizures, that is, generalized tonic–clonic convulsions with loss of righting reflexes.

Isolation of mitochondria

The animals were killed by decapitation on day 21 of PTX treatment and the brains were quickly dissected out and placed in beakers containing chilled (0–4°C) isolation medium (0.25 M sucrose containing 10 mM Tris–HCL, pH 7.4, 1 mM EDTA, and 250 μg BSA/ml) (Katyare et al., 2003). The tissue was thoroughly washed with isolation medium to make it free from blood and 10% (w/v) homogenate was prepared using a Potter Elvehjem type glass-Teflon homogenizer. The nuclei and cell debris were sedimented by centrifugation at 650 × *g* for 10 min and discarded. The supernatant was subjected to a further centrifugation at 10,000 × *g* for 10 min. From the sedimented fraction the loosely packed synaptosomal-myelin fraction was discarded after gentle swirling, taking care not to disturb the tightly packed mitochondrial pellet. The mitochondria were washed by gently suspending them in isolation medium and resedimenting at 6500 × *g* for 10 min. Finally, the mitochondria were suspended in the isolation medium to give a protein concentration of ca.

10–15 mg/ml. All the steps in the isolation procedure were carried out at 0–4°C in a Sorvall RC 5Bplus refrigerated centrifuge using SS34 rotor. We have previously shown that this procedure yields pure mitochondrial preparations that are practically free from synaptosomal, microsomal, and cytosolic contaminations (Katyare et al., 1994).

Measurement of oxidative phosphorylation

Measurement of oxidative phosphorylation was carried out using Clark type oxygen electrodes as described previously (Katyare et al., 1994, 2003). The respiration medium contained, in a volume of 1.6 ml, 225 mM sucrose, 5 mM potassium phosphate buffer, pH 7.4, 10 mM Tris–HCl buffer, pH 7.4, 10 mM KCl, 0.2 mM EDTA, and 100 μg BSA/ml. Depending on the substrate used, 2–5 mg of mitochondrial protein was added and respiration was initiated by the addition of substrate. The substrates used were glutamate (10 mM), pyruvate (10 mM) + malate (1 mM), succinate (10 mM), and ascorbate (10 mM) + TMPD (0.1 mM). With the latter two substrate 0.1 μM rotenone was included. State 3 respiration rates were initiated by the addition of about 75–150 nmol of ADP in small aliquots (10–20 μl) and the respiration rates in the presence of added ADP (state 3) and after its depletion (state 4) were recoded. Calculations of the ADP/O ratio and ADP phosphorylation rates were as described previously (Katyare et al., 1977; Shukla et al., 2000).

Assay of dehydrogenases

Malate dehydrogenase (MDH) activity was measured in the assay medium containing, in a total volume of 1 ml, 120 mM potassium phosphate buffer, pH 7.8, 2.5 mM sodium oxaloacetate, 0.1% Triton X-100, and 10–20 μg of mitochondrial protein as the source of enzyme. After preincubation at 37°C for 1 min, the reaction was initiated by the addition of 1 mM NADH. The liner rate of reaction was recorded at 5-s intervals by monitoring the decrease in absorbance at 340 nm in a Shimadzu UV 160 spectrophotometer (Ochoa, 1955).

Succinate dehydrogenase (SDH, succinate DCIP reductase) activity was measured spectrophotometrically in the assay medium (total volume 1 ml) containing 120 mM potassium phosphate buffer, pH 7.4, 1.5 mM freshly prepared KCN, 20 mM sodium succinate, and 200 μg mitochondrial protein as the source of the enzyme. After incubation at 37°C for 1 min, the reaction was initiated by the addition of 10 μM DCIP and the decrease in absorbance at 600 nm was recorded at 5-s intervals (King, 1967).

Assay of ATPase

The ATPase activities were determined in the assay medium (total volume 0.1 ml) containing 250 mM sucrose, 10 mM Tris–HCl buffer, pH 7.4, 10 mM KCl, 0.2 mM EDTA. The assays were performed in the absence and presence of $MgCl_2$ (2 mM) and DNP (50 μM), or a combination thereof. After preincubating the mitochondrial protein (ca. 50–70 μg) in the assay medium at 37°C, the reaction was initiated by addition of ATP at a final concentration of 2 mM. The reaction was carried out for 10 min and then terminated by the addition of 1.1 ml of 5% (w/v) trichloroacetic acid (TCA). The amount of liberated inorganic phosphorous was estimated by the method of Katewa and Katyare (2003).

Substrate and temperature kinetics

The kinetic studies were carried out in the assay medium described above containing both Mg^{2+} and DNP. For substrate kinetic studies, the concentration of ATP was varied from 0.04 to 2 mM. The temperature dependence of the enzyme activity was measured in the presence of fixed substrate concentration at 2 mM and the temperature was varied from 5 to 53°C (4°C steps). Analysis of substrate

Table 1
Effect of PTX-induced seizures on oxidative phosphorylation in rat brain mitochondria

Substrate	Animals	APD/O ratio	Respiration rate (nmol O_2/min/mg protein)		ADP phosphorylation rate (nmol/min/mg protein)
			+ADP	−ADP	
Glutamate	Cont (16)	3.49 ± 0.16	33.75 ± 1.36	12.83 ± 0.69	233.19 ± 12.44
	PTX (24)	3.18 ± 0.17	19.85 ± 0.59***	9.42 ± 0.46***	130.28 ± 9.46***
Pyruvate + Malate	Cont (16)	4.50 ± 0.21	32.04 ± 1.60	9.59 ± 0.62	284.47 ± 17.61
	PTX (24)	3.00 ± 0.07***	26.76 ± 1.34*	12.43 ± 0.58**	158.40 ± 7.82***
Succinate	Cont (16)	1.79 ± 0.11	55.95 ± 2.21	32.88 ± 1.77	228.82 ± 16.88
	PTX (24)	0.96 ± 0.07***	45.74 ± 2.42**	28.77 ± 1.66	80.85 ± 4.97***
Ascorbate + TMPD	Cont (16)	0.25 ± 0.01	43.06 ± 2.98	28.75 ± 2.62	21.83 ± 1.22
	PTX (24)	0.34 ± 0.02**	49.02 ± 3.33	30.14 ± 2.33	31.76 ± 2.14***

Note. The experimental conditions are as described in the text. The results are expressed as mean ± SEM of the number of observation indicated in the parentheses.
* $P < 0.025$.
** $P < 0.005$.
*** $P < 0.001$.

Table 2
Effect of PTX-induced seizures on rat brain mitochondrial dehydrogenases activities

Animals	Malate dehydrogenase (MDH)	Succinate dehydrogenase (SDH)
Control (12)	2610.94 ± 82.44	11.16 ± 0.57
PTX (20)	2058.50 ± 125.7*	2.19 ± 0.22*

Note. The experimental details are as given in the text. The enzyme activities are in nmol/min/mg protein. The results are given as mean ± SEM of the number of observation indicated in the parentheses.
* $P < 0.001$.

kinetics data for determination of K_m and V_{max} was according to Dixon and Webb (1979). For the temperature kinetics data, the determination of energies of activation for the high and low temperature ranges (E1 and E2, respectively) and phase transition temperature (T_t) were calculated from the Arrhenius plots (Raison, 1972). Analyses of the data were carried out by employing Sigma Plot, version 5.0 (Dave et al., 2000).

Cytochrome content

The contents of cytochromes (aa_3, b, and $c + c_1$) were calculated from the difference spectra as described previously (Katyare and Rajan, 1988; Poyton and Mc Ewen, 1996). Briefly, 8–10 mg mitochondrial protein was taken up in potassium phosphate-buffered sucrose and solubilized by adding 0.4 ml 10% Triton X-100. The total volume was made up to 2.5 ml. The sample was then transferred to two 1-ml cuvettes. The sample in the reference cuvette was oxidized by adding a small amount of potassium ferricyanide and the sample in the experimental cuvette was reduced by adding a few milligrams of sodium dithionite. From the difference spectra, contents of c + c_1, b, and aa_3 were calculated using the wavelength pairs 540–552, 562–575, and 605–625 nm, respectively (Katyare and Rajan, 1988; Poyton and Mc Ewen, 1996). The spectra were recorded in a Shimadzu UV 160 spectrophotometer.

Lipid analysis

Extraction of total lipids (Folch et al., 1957; Bangur et al., 1995), separation of phospholipid classes by thin layer chromatography (Skipiski et al., 1967), and estimation of cholesterol (CHL; Zlatkis et al., 1953) were by the methods cited. The phospholipid phosphorous was determined by the method of Katewa and Katyare (2003).

Membrane fluidity

Measurements on membrane fluidity were carried out with DPH as the probe in a Shimadzu RF 5000 spectrophotofluorimeter following the procedures described earlier in details (Bangur et al., 1995; Mehta et al., 1991).

Protein estimation was done by the method of Lowry et al. (1951) with BSA as the standard. Statistical evaluation of the data was performed using the Students' t test.

Results

The results of the effects of PTX-induced seizures on rat brain mitochondrial oxidative phosphorylation are summarized in Table 1. From the data presented, it can be noted that PTX treatment resulted in overall 27–41% inhibition of state 3 and state 4 respiration rates with glutamate as substrate. The ADP phosphorylation rate was lower by 44%. With pyruvate + malate as the substrate pair, the extent of inhibition of state 3 respiration rate was 16%, while state 4 respiration rate was higher by 1.3-fold. The ADP/O ratio was decreased by 33%. As a consequence, the ADP phosphorylation rate also decreased to a similar extent as observed for glutamate. When succinate was used as substrate, PTX treatment affected state 3 and state 4 respiration rates to a lesser extent and the inhibition ranged from 13% to 18%. However, the uncoupling of mitochondria was much higher compared to the former two substrates. Thus, the ADP/O ratio decreased by about 47%. The overall effect was a decrease in ADP phosphorylation rate by 65%. With ascorbate + TMPD as the substrate, the respiration rates were not affected after PTX treatment. However, the ADP/O ratio was higher by 1.3-fold. Consequently, the ADP phosphorylation rate was elevated by about 1.4-fold.

The effect of PTX-induced seizures on dehydrogenase activities is shown in Table 2. It is evident that malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities were lowered by 20% and 80%, respectively.

Since F_0F_1ATPase (complex V) plays an important role in ATP synthesis (Lehninger et al., 1993), the effect of PTX-induced epileptic condition on mitochondrial ATPase activity was estimated under different conditions. The results are presented in Fig. 1. As can be noted, in the controls, addition of Mg^{2+} shows stimulation of ATPase activity while DNP did not have any effect. PTX treatment

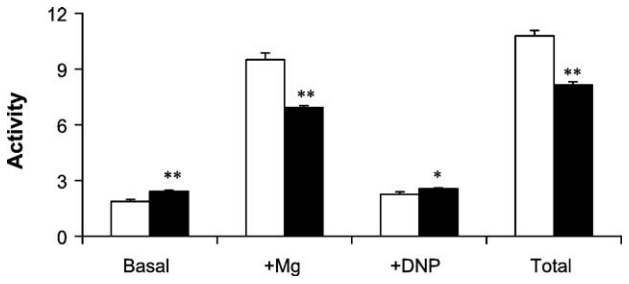

Fig. 1. Effect of PTX-induced seizures on rat brain mitochondrial ATPase activity. The experimental conditions are as described in text. The results are given as mean ± SEM of eight independent observations. The ATPase activity is given in μmol P_i liberated/h/mg protein. Control (□) and PTX treated (■). *$P < 0.05$ and **$P < 0.001$.

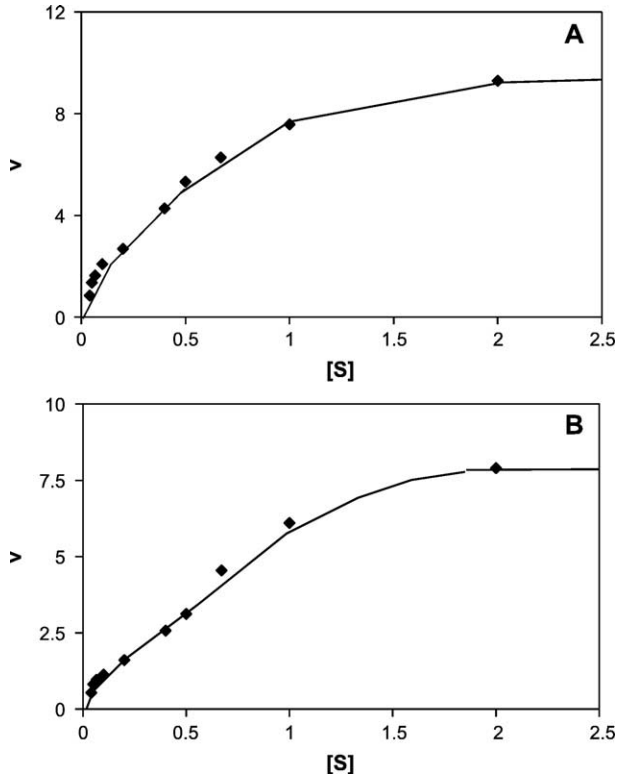

Fig. 2. Typical substrate saturation curves for rat brain mitochondrial ATPase in control (A) and PTX-treated (B) animals. The experimental details are as described in text. Concentration of ATP [*S*] ranged from 0.04 to 2 mM. The enzyme activity (*v*) is expressed as μmol P_i liberated/h/mg protein.

caused elevation of basal and DNP-stimulated activities, whereas Mg^{2+}-stimulated activity was lower. Therefore, composite decrease in total activity (+Mg^{2+} and +DNP) was observed.

The changes in ATPase activity suggest that the kinetic attributes of the enzyme might have altered. This possibility was verified by examining the substrate and temperature kinetics of the enzyme. The substrate saturation kinetics was considerably altered in the PTX-treated animals (Fig. 2). These data were then analyzed further by Lineweaver–Burk and Eadie–Hofstee plots. Typical Eadie–Hofstee plots are shown in Fig. 3. In both controls as well as PTX-induced epileptic groups, the mitochondrial ATPase activity could be resolved into two kinetic components. Component I had low K_m and V_{max}, while the opposite was true for component II. The calculated values of K_m and V_{max} from the Lineweaver–Burk and Eadie–Hofstee plots were in close agreement, thus averages of the pooled values are given in Table 3. It is evident that in the epileptic animals the V_{max} for component I was lower by 35% with a corresponding 36% decrease in K_m. While in the case of component II, the K_m values were about 2-fold higher.

In the next set of experiments, temperature dependence of the enzyme activity was monitored. It was observed that Arrhenius plots (Fig. 4) in the control group were biphasic with two energies of activation (E1 and E2) and a phase transition temperature (T_t). PTX-induced seizures resulted into 1.2-fold increase in both E1 and E2, while T_t was decreased by 4°C (Table 3).

The effect of PTX-induced convulsions on cytochrome content in the mitochondria was evaluated. The typical cytochrome spectra are shown in Fig. 5, and the contents of the respective cytochromes are given in Table 3. PTX treatment resulted in 20% decrease in cytochrome *b* content without any appreciable changes in cytochrome aa_3 and cytochrome $c + c_1$ content (Table 3).

Since mitochondrial respiratory chain (MRC) and F_0F_1 ATPase are membrane-bound moieties and any alterations in lipid/phospholipid environment can result into functional impairment of the system as a whole, hence the studies were extended to examine the effect of PTX-induced epileptic condition on the structural properties of mitochondrial membrane. These results are shown in Tables 3 and 4. The total phospholipid (TPL) and cholesterol (CHL) content decreased significantly by 15% and 17%, respectively, without affecting the TPL/CHL molar ratio (Table 3). Brain mitochondrial membrane was somewhat more fluidized under the epileptic condition (Table 3). The analysis of phospholipid composition revealed that lysophospholipid (lyso), sphingomyelin (SPM), and phosphatidylcholine (PC) components increased by 1.3- to 2-fold; while phosphati-

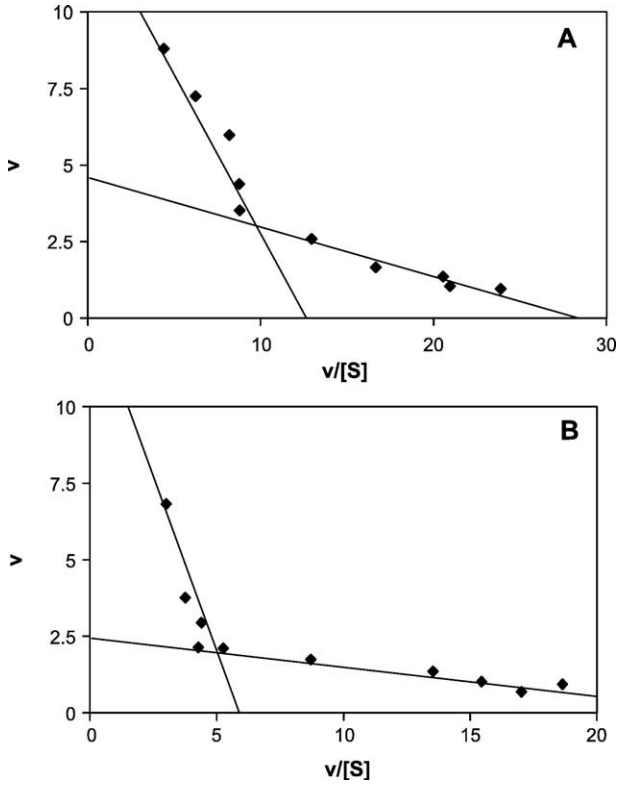

Fig. 3. Typical Eadie–Hofstee plots for rat brain mitochondrial ATPase in control (A) and PTX-treated animals (B). The experimental details are as described in text. Concentration of ATP was in the range of 0.04–2 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents the *v*/[*S*] ratios. Reaction velocity is in μmol P_i liberated/h/mg protein. *v*/[*S*] is reaction velocity divided by the corresponding substrate concentration.

Table 3
Effect of PTX-induced seizures on substrate and temperature kinetics properties of ATPase, cytochrome content, total phospholipid and cholesterol content, and membrane fluidity of rat brain mitochondria

	Parameter		Control	PTX
Substrate kinetics properties of ATPase	Component I	K_m	0.14 ± 0.004 (8)	0.09 ± 0.003 (8)***
		V_{max}	3.89 ± 0.097 (8)	2.51 ± 0.061 (8)***
	Component II	K_m	1.20 ± 0.051 (8)	2.56 ± 0.221 (8)***
		V_{max}	13.94 ± 0.51 (8)	15.27 ± 0.37 (8)***
Temperature kinetics properties of ATPase	Energy of activation (kJ/mol)	E1	42.04 ± 1.32 (8)	49.16 ± 2.01 (8)*
		E2	58.15 ± 1.46 (8)	64.84 ± 1.33 (8)*
	Phase transition temperature (°C)	T_t	23.67 ± 0.70 (8)	19.75 ± 0.60 (8)***
Cytochrome content (pmol/mg protein)	Cytochrome aa_3		222.21 ± 16.01 (16)	222.75 ± 10.08 (17)
	Cytochrome b		241.98 ± 12.41 (16)	192.54 ± 7.48 (17)**
	Cytochrome $c + c_1$		241.86 ± 16.13 (16)	220.34 ± 10.42 (17)
Total phospholipid	TPL (μg/mg protein)		555.43 ± 7.97 (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 ($r\alpha$)		0.093 ± 0.0042 (16)	0.073 ± 0.0039 (12)**
	Order parameter (S)		0.483 ± 0.0121 (16)	0.429 ± 0.0141 (12)**

Note. The experimental details are as given in the text. K_m is given as mM and V_{max} expressed as μmol of P_i liberated/h/mg protein. The results are given as mean ± SEM of the number of observation indicated in the parentheses.
* $P < 0.01$.
** $P < 0.002$.
*** $P < 0.001$.

dylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and diphosphatidylglycerol (DPG) were reduced by 17–78%, with maximum lowering observed for DPG (Table 4).

Discussion

Defects in oxidative phosphorylation in the central nervous system are the characteristic sign of mitochondrial encephalopathies, which are observed in variety of diseases with epileptic phenotype (Finsterer, 2004). The present investigations were initiated to find out the effects of chronic epileptic condition induced by PTX on brain mitochondrial function. Our results have clearly shown that the mitochondrial respiratory rates were inhibited by the PTX-induced epileptic condition. The major lesions were impairment of ADP/O ratios and respiration rates with pyruvate + malate, glutamate, and succinate as the respiratory substrates (Table 1). Interestingly, the extent of inhibition was relatively higher when succinate was used as substrate. This evidence is also supported by decreased activity of dehydrogenases (MDH and SDH) after PTX treatment (Table 2). These would suggest that the extent of inhibition was common for both NAD^+ and flavins-linked systems. The contrasting feature was elevated rates of respiration with ascorbate + TMPD as the electron donor. The possible explanation would be ascorbate + TMPD does not include any dehydrogenase systems. One interesting observation was that the mitochondrial oxidative phosphorylation was uncoupled in the epileptic condition, which can be easily derived from the data given in Table 1. It is worth to note in this context that expression of mitochondrial uncoupling protein 2 (UCP2) was increased during seizure activity that dissociates the cellular energy metabolism (Diano et al., 2003). Because decline of respiratory chain activities caused a rate limitation of mitochondrial respiration, it could also affect the generation of the mitochondrial membrane potential that is linked to respiration by proton pumping.

Analysis of the cytochrome content revealed that cytochrome b that is encoded by mitochondrial DNA (mtDNA) (Poyton and Mc Ewen, 1996) was significantly decreased under PTX-induced epileptic animals (Table 3). Interestingly, 2- to 3-fold decrease in mtDNA copy number has been reported in pilocarpine-induced convulsions (Kudin et al., 2002). mtDNA damage has also been reported in experimental models of epilepsy (Liang et al., 2000; Yamamoto and Mohanan, 2003). Possible mutation of mtDNA could also lead to decreased expression of mitochondrial-encoded subunits of respiratory chain. Multiple deficiencies of mitochondrial respiratory chain (MRC) complex I and IV and cytochrome oxidase have been documented for mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy with 'ragged red fibers' (MERRF) syndromes (Finsterer, 2004).

Additionally, the basal and DNP-stimulated ATPase activities had increased following PTX treatment (Fig. 1), which is the suggestive of increased mitochondrial membrane fragility. It is also evident from the data presented in Table 3 that PTX treatment resulted in altered substrate kinetics of ATPase. In fact, the K_m and V_{max} of component I, which is a high-affinity component, were found to be decreased; while that of component II was increased in the

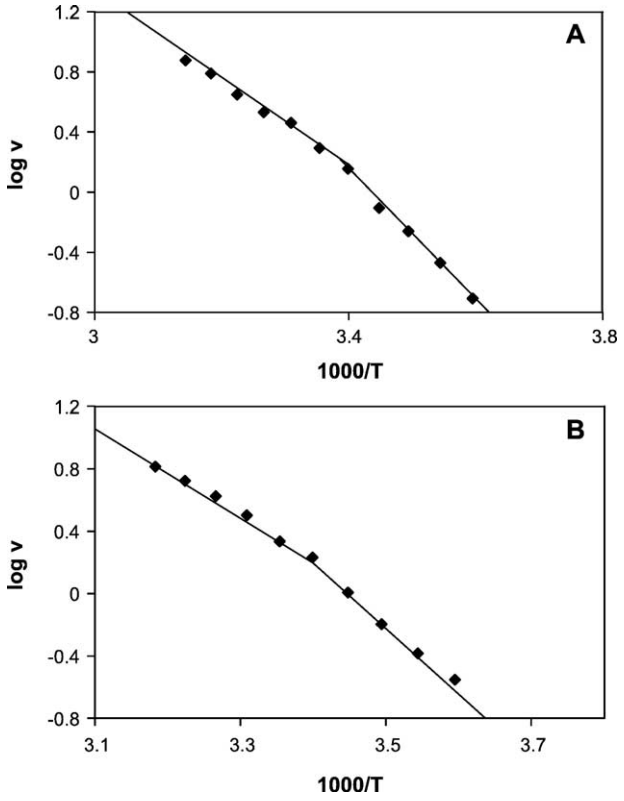

Fig. 4. Typical Arrhenius plots for rat brain mitochondrial ATPase in control (A) and PTX-treated (B) animals. The experimental details are as described in text. The abscissa represents the log of reaction velocity v, while the ordinate represents reciprocal of absolute temperature $T \times 1000$. Reaction velocity is expressed as μmol P_i liberated/h/mg protein and absolute temperature T in Kelvin.

epileptic condition. Likewise, Arrhenius plots also revealed significant alterations following PTX treatment (Fig. 4 and Table 3).

Requirement of phospholipids for the optimum functioning of mitochondrial membrane proteins is well documented (Daum, 1985). Our results on the mitochondrial membrane properties indicated drastic alterations in membrane composition (Table 4). ATPase activity is known to be dependent on acidic phospholipids, in particular PS and PI. Hence, observed changes in the kinetic parameters of ATPase could be attributed to altered phospholipid composition and distorted membrane charge distribution. Also, mitochondrial membrane became more fluidized under the epileptic condition (Table 3). Membrane fluidity is determined by several parameters including mole/mole ratios of TPL/CHL, SPM/PC, SPM/PE, and PC/PE (Bangur et al., 1995; Senault et al., 1990). Fluorescence polarization (P) was negatively correlated with PC/PE ratio (data not shown). It is also possible that substantial increase in lysophospholipid may lead to the fluidization of the membrane, since lysophosphoglycerides can exert detergent-like action on the membranes (Rossiter and Strickland, 1960). Increased lysophospholipids indicate activation of phospholipases, which are known to be activated by Ca^{2+} (Trimenstein and Nelson, 1989). Mitochondrial function is crucial to intracellular Ca^{2+} homeostasis and possesses several Ca^{2+} transport systems (Nicholls, 1985). 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 (White and Reynolds, 1995). However, chronic seizure activity could lead to modulation of aforementioned systems and thereby could activate the Ca^{2+}-dependent downstream pathways.

Significant alterations in ATPase activity and kinetic properties, which are observed here, could be an adaptation to the increased demand of energy during prolonged seizure activity. Decreased phosphate energy metabolism is suspected to play a particular role in seizure-related neuronal damage (Walton et al., 1999; Wasterlain et al., 1993). Activity of the synaptic Mg^{2+}, Ca^{2+}-dependent ecto-ATPase was also decreased in the epileptic condition (Nagy et al., 1997). When mitochondrial ATPase become compromised, a fall in cellular ATP levels results in decreased Ca^{2+} and Mg^{2+} pump activity and thus subsequent decrease in neuronal energy and increased membrane excitability could be implemented (Novelli et al., 1988; Thayer and Miller, 1990).

The changes in energy metabolism suggest that during seizure activity, residual production does not keep up with the increased energy demands. ATP is involved in many physiological processes such as maintaining the ion/cellular pumps, mitochondrial Ca^{2+} homeostasis, various phosphorylation processes, regulation in cell membrane permeability, protein and neurotransmitter biosynthesis, and exocytosis (Boyer, 1998). Nevertheless, significant decrease in nicotinamide adenine dinucleotide (NAD) in brain regions by kainate-induced seizures has been reported (Gupta and Dettbarn, 2003). For every mole of NAD that is consumed, four free energy equivalent of ATP are required to generate NAD. This high utilization of energy when coupled with inhibition of oxidative phosphorylation and dehydro-

Table 4
Effect of PTX-induced convulsions on phospholipid composition of rat brain mitochondria

Phospholipid class	Phospholipid composition (percentage 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

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

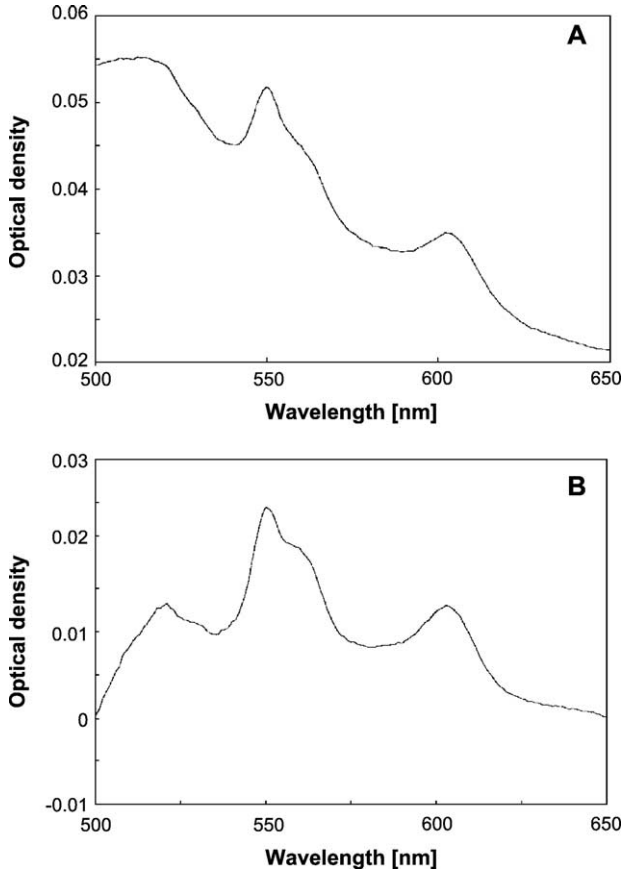

Fig. 5. Typical difference spectra of cytochromes of rat brain mitochondria from control (A) and PTX-treated (B) animals. The experimental details are as described in text.

genases, which is apparent here, compromises the cell's capability to maintain energy levels causing mitochondrial and neuronal damage. Neuropathological investigations have repeatedly pointed seizure-related alterations of neurons characterized by swollen and often disrupted mitochondria (Meldrum, 1993).

MRC is long being recognized as the major source of reactive oxygen species (ROS) in cells (Cadenas et al., 1977) and one of the most important being the production of highly damaging superoxide (O_2^-) and nitric oxide (NO). Mammalian mitochondria are sensitive targets for cytotoxic effects of ROS, especially because of higher PUFA content. NO is reported to specifically interact with mitochondrial function by competing with oxygen for cytochrome *c* oxidase, interacting with complex II and III to irreversibly blocking respiration and ATP synthesis (Brookes et al., 1999; Brown, 1999; Zhang et al., 1990). Complex I is extremely sensitive to oxidative stress because of irreversible oxidative modification of [4Fe-4S] clusters (Zhang et al., 1990). Similarly, mtDNA is known to be extremely vulnerable due to lack of protective histones and inadequate repair mechanism against oxidative damage (Suter and Richter, 1999). 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 (Bolanos et al., 1997; Cock, 2002; Gupta et al., 2001; Ueda et al., 1997). Likewise, increased ROS production and decreased scavenging enzyme activity have also been documented in long-term seizure activity (Candelario-Jalil et al., 2001; Cock, 2002; Li et al., 1998). Nevertheless, MRC dysfunction along with elevated free radicals could also trigger the cell death pathways in the epileptic condition (Cock, 2002; Kromer and Reed, 2000; Meldrum, 1993; Niquet et al., 2003).

In conclusion, our results on MRC dysfunction along with alterations in complex V kinetics and drastic variations in the mitochondrial membrane properties support the belief of possible role of mitochondria in epileptogenesis. Impaired MRC activity that has consequences on neuronal energy metabolism suggests novel pathophysiological mechanisms important for chronic epileptic condition. Additionally, mitochondrial dysfunction could directly affect the phosphorylation potential-dependent Na^+/K^+-ATPase and thus cause a lowered resting membrane potential at the plasma membrane, which could contribute to hyperexcitability and decreased threshold for long-term seizure activity.

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References

Acharya, M.M., Khamesra, S.H., Katyare, S.S., 2005. Picrotoxin-induced convulsions and lysosomal function in rat brain. Ind. J. Clin. Biochem. 20 (1), 56–60.

Astrup, J., Sorensen, P.M., Sorensen, H.R., 1981. Oxygen and glucose consumption related to Na^+-K^+ transport in canine brain. Stoke 12, 726–730.

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

Bolanos, J.P., Almeida, A., Stewart, S., et al., 1997. Nitric-oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. J. Neurochem. 68, 2227–2240.

Boyer, P.D., 1998. Energy, life and ATP. Biosci. Rep. 18, 97–117.

Brookes, P.S., Bolanos, J.B., Heales, S.J., 1999. The assumption that nitric oxide inhibits mitochondrial ATP synthesis is correct. FEBS Lett. 446, 261–263.

Brown, G.C., 1999. Nitric oxide and mitochondrial respiration. Biochim. Biophys. Acta 1411, 351–369.

Cadenas, E., Boveris, A., Ragan, C.I., Stoppani, A.O.M., 1977. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef heart mitochondria. Arch. Biochem. Biophys. 180, 248–257.

Candelario-Jalil, E., Al-Dalain, S.M., Castillo, R., et al., 2001. Selective vulnerability to kainate-induced oxidative damage in different rat brain regions. J. Appl. Toxicol. 21 (5), 403–407.

Canfoglia, L., Franceschetti, S., Antozzi, C., et al., 2001. Epileptic phenotype associated with mitochondrial disorders. Neurology 56, 1340–1346.

Cock, H.R., 2002. The role of mitochondria and oxidative stress in neuronal damage after brief and prolonged seizures. Prog. Brain Res. 135, 187–196.

Daum, G., 1985. Lipids of mitochondria. Biochem. Biophys. Acta 822, 1–42.

Dave, K.R., Syal, A.R., Katyare, S.S., 2000. Tissue cholinesterase. A comparative study of their kinetic properties. Z. Naturforsch. 55c, 100–108.

Diano, S., Matthews, R.T., Patrylo, P., et al., 2003. Uncoupling protein 2 prevents neuronal death including that occurring during seizures: a mechanism for preconditioning. Endocrinology 144 (11), 5014–5021.

DiMurro, S., Kulikova, R., Tanji, K., et al., 1999. Mitochondrial genes for generalized epilepsies. Adv. Neurol. 79, 411–419.

Dixon, M., Webb, C., 1979. Enzymes. Longmann, London, pp. 47–206.

Fisher, R.S., 1989. Animal models of epilepsies. Brain Res. Rev. 14, 245–278.

Finsterer, J., 2004. Mitochondriopathies. Eur. J. Neurol. 11, 163–186.

Folbergrova, J., Ingvar, M., Nevander, G., Siesjo, B.K., 1985. Cerebral metabolic changes during and following fluorothyl-induced seizures in ventilated rats. J. Neurochem. 44, 1419–1426.

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

Fujikawa, D.G., Shinmei, S.S., Cai, B., 2000. Kainic acid-induced seizures produce necrotic, not apoptotic, neurons with internucleosomal DNA cleavage: implications for programmed cell mechanisms. Neuroscience 98, 41–53.

Green, D.R., Reed, J.C., 1988. Mitochondria and apoptosis. Science 281, 1309–1310.

Gupta, R.C., Dettbarn, W.-D., 2003. Prevention of kainic acid seizures-induced changes in levels of nitric oxide and high-energy phosphates by 7-nitroindazole in rat brain regions. Brain Res. 981, 184–192.

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

Herrington, J., Park, Y.B., Babcock, D.F., Hille, B., 1996. Dominant role of mitochondria in clearance of large Ca^{2+}-loads from rat adrenal chromaffin cells. Neuron 16, 219–228.

Jope, R.S., Morrisett, R.A., 1986. Neurochemical consequences of status epilepticus induced in rats by coadministration of lithium and pilocarpine. Exp. Neurol. 93, 404–414.

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

Katyare, S.S., Rajan, R.R., 1988. Enhanced oxidative phosphorylation in rat liver mitochondria following prolonged in vivo treatment with imiparmine. Br. J. Pharmacol. 95, 914–922.

Katyare, S.S., Joshi, M.V., Fatterpaker, P., Sreenivasan, A., 1977. Effect of thyroid deficiency on oxidative phosphorylation in rat liver, kidney and brain mitochondria. Arch. Biochem. Biophys. 182, 155–163.

Katyare, S.S., Bangur, C.S., Howland, J.H., 1994. Is respiratory activity in the brain mitochondria responsive to thyroid hormone action? A critical re-evaluation. Biochem. J. 302, 857–860.

Katyare, S.S., Balasubramanian, S., Parmar, D.V., 2003. Effect of corticosterone treatment on mitochondrial oxidative energy metabolism in developing rat brain. Exp. Neurol. 183/1, 241–248.

King, T.E., 1967. Preparation of succinate cytochrome *c* reductase and the cytochrome *b*–*c* particle and the reconstitution of succinate cytochrome *c* reductase. In: Estabrook, R.W., Pullman, M.E. (Eds.), Methods Enzymol., vol. X. Academic Press, New York, pp. 216–225.

Kobayashi, S., Kikuchi, H., Ishikawa, M., Hashimoto, K., 1990. Regional changes of tissue pH and ATP content in rat brain following systemic administration of kainic acid. Brain Res. 514, 352–354.

Kromer, G., Reed, J.C., 2000. Mitochondrial control of cell death. Nat. Med. 6, 513–519.

Kubova, M., Bohuslav, T., Mares, P., 1992. Effects of clonazepam on picrotoxin induced convulsions. Physiol. Res. 41, 167–169.

Kudin, A.P., Tatiana, A.K., Seyfried, J., et al., 2002. Seizure-dependent modulation of mitochondrial oxidative phosphorylation in rat hippocampus. Eur. J. Neurosci. 15, 1105–1114.

Kunz, W.S., Kudin, A.P., Vielhaber, S., et al., 2000. Mitochondrial complex I deficiency in the epileptic focus of patients with temporal lobe of epilepsy. Ann. Neurol. 48, 766–773.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 1993. Biochemistry, Second ed. Worth Publishers, New York, pp. 542–571.

Li, Y., Copin, J.C., Reola, L.F., et al., 1998. Reduced mitochondrial manganese-superoxide dismutase activity exacerbates glutamate toxicity in cultured mouse cortical neurons. Brain Res. 814 (1–2), 164–170.

Liang, L.P., Ho, Y.S., Patel, M., 2000. Mitochondrial superoxide production in kainate-induced hippocampal damage. Neuroscience 101, 563–570.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mazarati, A.M., Halaszi, E., Telegdy, G., 1992. Anticonvulsant effects of galanin administrated into the central nervous system upon the picrotoxin-induced seizures syndrome in rats. Brain Res. 589, 164–166.

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

Meldrum, B.S., 1993. Excitotoxicity and selective neuronal loss in epilepsy. Brain Pathol. 3, 405–412.

Nagy, A.K., Walton, N.Y., Trieman, D.M., 1997. Reduced cortical Ecto-ATPase activity in rat brains during prolonged status epilepticus induced by sequential administration of lithium and pilocarpine. Mol. Chem. Neuropathol. 31, 135–147.

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

Niquet, J., Baldwin, R.A., Suni, A.G., Fujikawa, D.G., Waserlain, C.G., 2003. Hypoxic neuronal necrosis: protein synthesis independent activation of cell death program. Proc. Natl. Acad. Sci. 100, 2825–2830.

Novelli, A., Reilly, J.A., Lysko, P.G., Heneberry, R.C., 1988. Glutamate becomes neurotoxic via the *N*-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res. 451, 205–212.

Ochoa, S., 1955. Malic dehydrogenase in pig heart. In: Colowick, S.P., Kalpon, N.C. (Eds.), Methods Enzymol., vol. I. Academic Press, New York, pp. 735–739.

Poyton, R.O., Mc Ewen, J.E., 1996. Crosstalk between nuclear and mitochondrial genomes. Annu. Rev. Biochem. 65, 563–607.

Raison, J.K., 1972. The influence of temperature-induced phase changes on the kinetics of respiration and other membrane-associated enzyme systems. Bioenergetik 4, 559–583.

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

Schuchmann, S., Buchheim, K., Meierkord, H., Heinmann, U., 1999. A relative energy failure is associated with low Mg^{2+} but not with 4-aminopyridine induced seizure-like events in entorhinal cortex. J. Neurophysiol. 81, 399–402.

Schwarcz, R., Zaczek, R., Coyle, J.T., 1978. Microinjection of kainic acid into the rat hippocampus. Eur. J. Pharmacol. 50, 209–220.

Senault, C., Yazbeck, J., Goubern, M., Portet, R., Vincent, M., Gallay, J., 1990. 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 1023, 283–288.

Shukla, V.H., Dave, K.R., Katyare, S.S., 2000. Effect if catecholamine depletion on oxidative energy metabolism in rat liver, brain and heart mitochondria: use of reserpine. Comp. Biochem. Physiol., C: Pharmacol. Toxicol. 121, 79–90.

Skipiski, V.P., Barelay, M., Barelay, R.K., et al., 1967. Lipid composition of human serum lipoprotein. Biochem. J. 104, 340–361.

Suter, M., Richter, C., 1999. Fragmented mitochondrial DNA is the predominate carrier of oxidized DNA bases. Biochemistry 38, 459–464.

Thayer, S.A., Miller, R.J., 1990. Regulation of intracellular free calcium concentration in single rat dorsal rood ganglion neurons in vitro. J. Physiol. (London) 425, 85–115.

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

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

Van den Pol, A.N., Obrietan, K., Belousov, A., 1996. Glutamate hyperexcitability and seizure-like activity throughout the brain and spinal cord upon relief from chronic glutamate receptor blockade in culture. Neuroscience 74, 653–674.

Waagepetersen, H.S., Sonnewald, U., Schousboe, A., 1999. The GABA paradox: multiple roles as metabolite, neurotransmitter, and neurodifferentiative agent. J. Neurochem. 73, 1335–1342.

Walton, N.Y., Gunawan, S., Treiman, D.M., 1990. Brain amino acid concentration changes during status epilepticus induced by lithium and pilocarpine. Exp. Neurol. 108, 61–70.

Walton, N.Y., Nagy, A.K., Treiman, D.M., 1999. Altered residual ATP content in rat brain cortex subcellular fractions following status epilepticus induced by lithium and pilocarpine. J. Mol. Neurosci. 11, 233–242.

Wasterlain, C.G., Fujikawa, D.G., Penix, L., Sankar, R., 1993. Pathophysiological mechanisms of brain damage from status epilepticus. Epilepsia 34, S37–S53.

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

Williams, J.M., Thompson, V.L., Mason-Parker, S.E., et al., 1998. Synaptic activity-dependent modulation of mitochondrial gene expression in the rat hippocampus. Mol. Brain Res. 60, 50–56.

Yamamoto, H.A., Mohanan, P.V., 2003. Effect of alpha-ketoglutarate and oxaloacetate on brain mitochondrial DNA damage and seizures induced by kainic acid in mice. Toxicol. Lett. 143 (2), 115–122.

Zhang, Y., Marcillat, O., Giulivi, C., et al., 1990. The oxidative inactivation of mitochondrial electron chain components and ATPase. J. Biol. Chem. 265, 16330–16336.

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