Biochemical Pharmacology, Vol. 38, No. 21, pp. 3835–3841, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

FUNCTIONAL CHANGES OF BRAIN MITOCHONDRIA DURING EXPERIMENTAL HEPATIC ENCEPHALOPATHY

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(*Received* 5 *October* 1988; *accepted* 27 *March* 1989)

Abstract—Several functional parameters were studied in a non-synaptic population of brain mitochondria from rats made cirrhotic by chronic treatment with carbon tetrachloride, with and without coma produced by a single injection of ammonium acetate. The following changes were observed in mitochondria from cirrhotic rats, independently of the presence of coma: (a) a large decrease in oxygen consumption with pyruvate-malate as substrate, but not with succinate, in both states 3 and 4; (b) a modified volume oscillation pattern, characterized by a notable diminution in the amplitude of the oscillation; (c) an altered pattern of acyl groups, with a decrease in the proportion of unsaturated with respect to saturated fatty acids. The following parameters were also measured in brain mitochondria from the cirrhotic rats and were found unchanged: (a) malate dehydrogenase and ATPase activities; (b) content of cytochromes; (c) phospholipid composition; (d) total fatty acid content. The possible significance of the changes observed is discussed in terms of the membranal and biochemical alterations that may be involved in the mechanism of hepatic encephalopathy.

Hepatic encephalopathy (HE)† may occur during acute or chronic liver failure and is characterized by behavioral abnormalities, deficiencies in memory and attention, distal tremor, extrapyramidal signs, progressive impairment of consciousness and, finally, coma [1]. The pathogenesis of this syndrome is still unknown. Among the various factors that have been proposed to be involved in HE are impairment of cerebral bioenergetic function [2] and alterations of neuronal membrane composition [3].

Chronic treatment with carbon tetrachloride (CCl_4) produces hepatic failure, and the subsequent administration of ammonium results in coma [4, 5]. In the present work we have used this experimental model to study several functional parameters of brain mitochondria during liver cirrhosis and HE. We have measured the activity of some mitochondrial enzymes linked to oxidative phosphorylation, as well as the mitochondrial cytochrome content, oxygen consumption, volume oscillation, and lipid composition.

MATERIALS AND METHODS

Experimental groups. Male Wistar rats, weighing 90–100 g, were used. Liver cirrhosis was induced by the intraperitoneal administration of 0.15 ml of CCl_4 in vegetable oil (1:6, v/v) three times weekly for 2 months, for a total of twenty doses. Coma, defined in behavioral terms as an unresponsive state with a complete loss of reflexes and of muscular tone, was induced by a single dose of ammonium acetate (5.2 mmol/kg) administered 24 hr after the last injection of CCl_4 [6]. Each of the four experimental groups of animals received one of the following: (a) vegetable oil + sodium acetate (control); (b) vegetable oil + ammonium acetate; (c) CCl_4 + sodium acetate; or (d) CCl_4 + ammonium acetate. At the time of coma, animals were killed, and brain, liver and blood samples were taken for the assays described below.

Preparation of brain mitochondria. Free, non-synaptic mitochondria, obtained by the Ficoll gradient procedure described by Lai and Clark [7], were used for all determinations. Free mitochondria were obtained from four pooled forebrains (except olfactory bulbs) after homogenization in 0.32 M sucrose containing 1 mM potassium–EDTA and 10 mM Tris–HCl, pH 7.4. The Ficoll used for the gradients was dialyzed overnight against glass-distilled water, and the density of the Ficoll solution was determined using a specific-gravity bottle. The final mitochondrial fraction was resuspended in the homogenization medium after being washed twice with albumin-containing medium. The yield routinely obtained was 3–5 mg of mitochondrial protein per g of tissue.

Submitochondrial particles were prepared from the mitochondrial fraction according to Racker and Horstmann [8]. The fraction was sonicated in a Durham sonicator at 4° for three periods of 2 min with 1-min intervals. After one centrifugation at 10,000 *g* for 15 min to sediment the intact mitochondria, the supernatant fraction was centrifuged at 45,000 *g* for 30 min to obtain the submitochondrial particles.

Enzyme assays. All assays were carried out spectrophotometrically at 25°. Triton X-100 (0.1% final concentration) was present in the assays to ensure maximal enzyme activity. Lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (EC 1.1.1.37) were estimated by following NADH oxi-

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† Abbreviations: HE, hepatic encephalopathy; and DNP, 2,4-dinitrophenol.

dation at 340 nm [9]. Acetylcholinesterase (EC 3.1.1.7) was assayed colorimetrically by the method of Ellman *et al.* [10]. ATPase (ATP phosphohydrolase, EC 3.6.1.3) was measured in the submitochondrial particles in an ATP-generating medium, according to Pullman *et al.* [11].

Mitochondrial respiration. Oxygen uptake was measured polarographically with an oxygen-sensitive electrode, as described by Clark and Nicklas [12], in a medium containing 225 mM mannitol, 75 mM sucrose, 5 mM phosphate/Tris, 10 mM Tris–HCl, 0.05 mM potassium–EDTA and 5 mM KCl, pH 7.4. State 3 conditions were initiated by the addition of 0.5 mM ADP in the presence of the substrates indicated in Results. The respiratory control ratio was the ratio of state 3/state 4 [13]. NADH (16 mM) was added to the mitochondrial and submitochondrial fractions in order to corroborate the integrity of the former and the metabolic activity of the latter.

Cytochrome measurements. Cytochromes *c*, *b* and aa_3 were detected at room temperature in an Aminco–Chance dual wavelength spectrophotometer. Samples were mitochondrial fractions subjected to one freeze–thaw cycle and reduced by the addition of 10 mM sodium hydrosulfite (dithionite). The extinction coefficient for each cytochrome was taken from Wilson and Epel [14].

Mitochondrial oscillations. Periodic changes in mitochondrial volume were followed by continuous measurement of light absorption at 650 nm at room temperature, according to the method described by Packer *et al.* [15], in a medium containing about 1 mg mitochondrial protein, 100 mM sucrose, 10 mM Tris–HCl, 0.3 mM Tris–EDTA and 10 mM succinate, pH 8. Other additions are indicated in Results.

Lipid components. Lipid constituents were extracted by the method of Folch *et al.* [16]. The phospholipids cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, together with phosphatidic acid, were separated according to the procedure of García-Sáinz and Fain [17], and after hydrolysis phosphate was measured by the method of Ames [18].

Methyl esters of fatty acids from lipidic extracts were prepared for gas–liquid chromatography, as described by Huang and Sun [19], and analyzed with a Hewlett–Packard 7620A chromatograph equipped with dual flame ionization detectors. Methyl esters were separated on a 183 cm × 0.2 cm glass column packed with 10% SP-2330 on 100/120 chromorob W AW 1-1851 (Supelco, Inc., Bellefonte, PA, U.S.A.). The operating column temperature was 195°. Helium, the carrier gas, was delivered at a rate of 35 ml/min at a pressure of 40 psi. Peaks of fatty acids were identified by comparison with standards (Sigma) and quantified with the help of a Hewlett–Packard integrator.

Electron microscopy. The resuspended mitochondrial fraction was fixed in 1% glutaraldehyde. After 2 hr, the samples were centrifuged at 9000 *g* for 5 min, and the pellet was washed with Milloning medium [20], postfixed with 2% OsO_4 in Milloning medium, dehydrated stepwise with ethanol and propylene oxide, and embedded in Epon. Sections were cut on an LKB ultramicrotome, and stained with uranyl acetate and lead citrate.

Determination of serum metabolites. Serum glutamic-pyruvic transaminase was determined according to Reitman and Frankel [21]; conjugated and unconjugated bilirubin were measured as described by Malloy [22] and albumin by the method of Garnall *et al.* [23].

Protein was determined by the method of Lowry *et al.* [24].

RESULTS

Liver function. Chronic CCl_4 treatment resulted in liver failure manifested by notable increases in liver weight, serum glutamic-pyruvic transaminase and bilirubin, and decreased serum albumin. The changes in these parameters were quantitatively very similar to those previously described [4, 5] and, therefore, are not shown here. The cirrhotic rats that received ammonium acetate showed the symptoms of coma, mentioned under Materials and Methods, 16–26 min after injection.

Mitochondrial fraction. The purity of the brain mitochondrial fraction was examined by electron microscopy. The fraction was enriched in well preserved mitochondria and essentially free from synaptosomes and myelin, confirming the results reported by Lai and Clark [7].

The distribution of some marker enzymes in the mitochondrial fraction in the four experimental groups is shown in Table 1. Whereas the concentrations of lactate dehydrogenase and acetylcholinesterase were low, malate dehydrogenase, as expected, was concentrated in this fraction. This pattern of enzyme distribution was not altered by CCl_4 or NH_4 treatment.

Substrate oxidation. Respiration of brain mitochondria from the four groups of experimental animals is summarized in Table 2. With pyruvate-malate as substrate, the mitochondria derived from CCl_4-treated rats, both non-comatose and comatose, showed a notable decrease of oxygen consumption in both state 4 and state 3, as compared with the non-cirrhotic rats. This inability to oxidize pyruvate-malate was evident even in the presence of the uncoupler 2,4-dinitrophenol (DNP). Nevertheless, the stimulation of respiration during state 3 was of a similar magnitude in the treated and control groups and, consequently, the respiratory control did not change. In contrast to the differences observed with pyruvate-malate, when succinate was added as substrate, oxygen consumption was not affected in the cirrhotic animals.

ATPase activity. This enzyme was measured in the submitochondrial particles. No differences were observed between CCl_4-treated and control rats or between comatose and non-comatose rats. The values were (μmol/min/mg protein): oil + Na^+, 1.8 ± 0.3; oil + NH_4^+, 1.7 ± 0.4; CCl_4 + Na^+, 1.7 ± 0.2; CCl_4 + NH_4^+, 1.5 ± 0.3 (mean ± SE, N = 4).

Cytochrome content. The concentration of cytochromes found was similar to that reported [7] for non-synaptic mitochondria. No changes were observed in the cytochrome content of mitochondria

Table 1. Enzyme distribution in brain mitochondria from control and cirrhotic rats

Treatment	Enzyme activities (μmol/min/mg protein)									Protein (mg/fraction)	
	LDH			AChE			MDH				
	H	M	M/H	H	M	M/H	H	M	M/H	H	M
Oil + sodium acetate	0.63	0.13	0.21	0.11	0.02	0.24	2.28	14.8	6.50	947	16
Oil + ammonium acetate	0.70	0.16	0.23	0.10	0.02	0.18	2.44	15.3	6.28	960	18
CCl_4 + sodium acetate	0.62	0.14	0.23	0.12	0.02	0.20	2.21	14.1	6.70	953	17
CCl_4 + ammonium acetate	0.65	0.16	0.25	0.11	0.02	0.17	2.40	14.8	6.20	958	17

Abbreviations: H, homogenate; M, mitochondrial fraction; LDH, lactate dehydrogenase; AChE, acetylcholinesterase; and MDH, malate dehydrogenase. Values are means of four experiments; the SE was, in all cases, less than 12%.

Table 2. Respiration of brain mitochondria from control and cirrhotic rats

Treatment	Respiration (natoms O/mg protein/min)				
	ADP/O	State 4	State 3	RCR	DNP
	With pyruvate-malate (5 mM–2.5 mM)				
Oil + sodium acetate	2.9	13	69	5.2	114
Oil + ammonium acetate	2.8	15	65	4.3	112
CCl_4 + sodium acetate	2.7	2	10	5.0	42
CCl_4 + ammonium acetate	2.8	2	9	4.6	48
	With succinate (10 mM)				
Oil + sodium acetate	1.9	34	93	2.7	121
Oil + ammonium acetate	1.8	39	96	2.5	117
CCl_4 + sodium acetate	1.8	30	84	2.8	126
CCl_4 + ammonium acetate	1.9	42	109	2.6	122

State 3 conditions were initiated by the addition of 0.5 mM ADP. RCR = respiratory control ratio. The 2,4-dinitrophenol concentration was 0.3 mM. Values are means of four independent experiments; the SE was, in all cases, less than 12%.

during the HE or the comatose state. The ratio of cytochromes $c:b:aa_3$ was 2.50:1.04:1.00 in all experimental groups.

Mitochondrial volume oscillation. Oscillation of mitochondrial volume resembles a damped harmonic oscillator, and several parameters have been established to describe it [15]. These parameters, obtained from traces such as those of Fig. 1, which were initiated by the addition of succinate, are shown in Table 3. It can be observed that in mitochondria from cirrhotic rats there was a diminution (44%) in expansion and a notable decrease in contraction (84%), as compared to control mitochondria. This pattern was reflected in a 100% increase in damping factor in the CCl_4-treated rats.

Phospholipid composition. The phospholipid composition of brain mitochondria is shown in Fig. 2. This composition was similar to that of mitochondria from other tissues, both excitable [25] and non-excitable [26], and it was not altered in the cirrhotic rats.

Acyl group composition. The fatty acids found in the cerebral mitochondria of control groups were similar to those previously reported [27]. The more abundant acyl groups detected were 16:0, 18:1, 18:0, 22:6 and 20:4, and the ratio of unsaturated/saturated fatty acids was 1.38 (Table 4). These fatty acids accounted for more than 90% of the total identified acyl groups. Although the total acyl group content did not change, the mitochondrial fatty acid pattern of cirrhotic rats, both comatose and non-comatose, was altered profoundly. The two more abundant unsaturated fatty acids, 18:1 and 22:6, decreased, while the saturated fatty acids increased. As a consequence, the unsaturated/saturated ratio decreased by 33% and 38% in non-comatose and comatose cirrhotic rats respectively.

DISCUSSION

The experimental model of HE in the rat used in this work has been shown to be equivalent to the human portal-systemic encephalopathy [6]. In fact, the hepatic functional tests and the induction of coma by NH_4 in the CCl_4-treated rats are similar to the clinical symptoms described in cirrhotic patients [1].

Alterations in the cerebral energy-related metabolism have been associated with HE [2]. Consistently with this proposal, in HE the cerebral rate of oxygen consumption and the cerebral blood flow decline roughly in parallel with the severity of the neurological alterations [28]. Although the changes in whole brain ATP and phosphocreatine are modest, failures in glycolysis and the tricarboxylic acid cycle

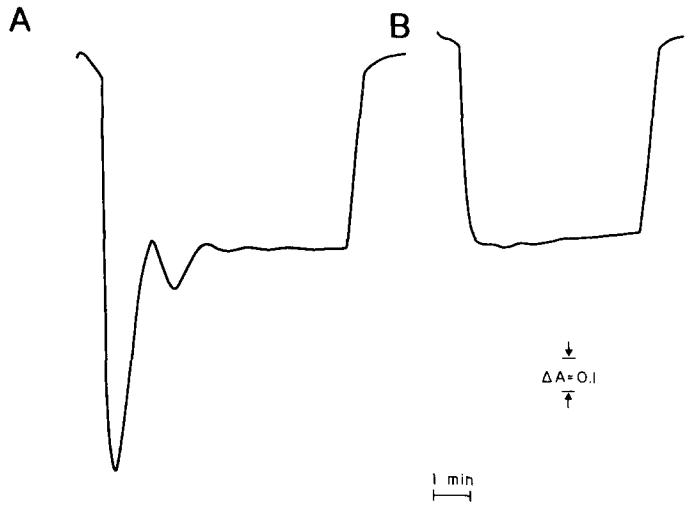

Fig. 1. Oscillations of brain mitochondria from control (A) and cirrhotic (B) rats. A loss of the oscillation pattern was observed in the CCl_4-treated animals, irrespective of the occurrence of coma. The traces illustrate the oscillation patterns observed in four experiments.

Table 3. Modifications of brain mitochondrial oscillation in cirrhotic rats

Treatment	Damping factor	Amplitude of mitochondrial oscillation ($\Delta A_{650} \times 10^3$/mg protein)	
		Expansion	Contraction
Oil + sodium acetate	7.7 ± 2.1	34 ± 4	22 ± 4
Oil + ammonium acetate	6.9 ± 1.0	32 ± 5	21 ± 4
CCl_4 + sodium acetate	15.3 ± 2.2*	20 ± 4*	4 ± 2*
CCl_4 + ammonium acetate	16.8 ± 2.7*	22 ± 2*	3 ± 1*

The criteria used for evaluation of oscillation parameters were according to Packer *et al.* [15].
Results are means ± SE of four experiments.
* $P < 0.05$ as compared to controls.

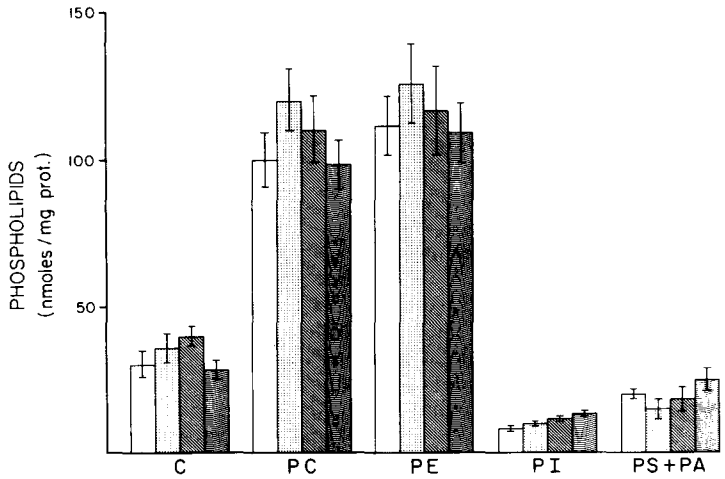

Fig. 2. Mitochondrial phospholipids in brain of control, cirrhotic and comatose rats. Abbreviations: C, cardiolipine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS + PA, phosphatidylserine plus phosphatidic acid. Key: (□) vegetable oil + sodium acetate; (▨) vegetable oil + ammonium acetate; (▩) CCl + sodium acetate (cirrhotic); and (▤) CCl_4 + ammonium acetate (comatose). The results are mean values of four experiments ± SE.

Table 4. Mitochondrial fatty acid composition in brain of control and cirrhotic rats

Treatment	Total fatty acids (μg/mg protein)	Composition (%)							Ratio Unsaturated / Saturated
		16:0	18:0	18:1	18:2	20:4	22:4	22:6	
Oil + sodium acetate	31 ± 4	22.2	19.9	20.7	2.0	12.8	2.7	18.5	1.38
Oil + ammonium acetate	29 ± 3	21.8	20.4	20.1	2.6	12.9	2.4	18.9	1.37
CCl_4 + sodium acetate	25 ± 3	24.3	27.8	14.1	2.2	12.9	2.0	15.7	0.92
CCl_4 + ammonium acetate	28 ± 4	25.3	28.6	13.6	2.3	13.5	2.9	15.0	0.86

Total fatty acids values are means ± SE, N = 4–6. Composition is presented as mean relative percentages determined from at least four different samples from each experimental group. Fatty acids are designated by the number of carbon atoms followed by the number of double bonds.

seem to occur as a consequence of the chronic high levels of ammonium which are characteristic of this syndrome [29]. In spite of this, the possibility that such alterations may be associated with functional disturbances of brain mitochondria during HE has not been explored previously.

Several populations of brain mitochondria have been described, differing in their location (synaptic, neuronal or glial), morphology [30] and enzyme content [31]. The non-synaptic mitochondria used in the present investigation, most probably derived from neuronal and glial somas [7], were metabolically active and well coupled, both with pyruvate and malate as substrates for site I and with succinate for site II (Table 2). The mitochondrial fraction used was also capable of oxidizing other substrates such as citrate, glutamate, α-oxoglutarate and acetate (data not shown), as previously reported [7].

In control rats the respiratory parameters with substrates for sites I and II were similar to those previously reported [7]. The decreased respiration observed in the mitochondria from the cirrhotic rats, when pyruvate-malate was used as substrate, may be explained either by a failure in the transport of the substrates into the mitochondria or by an impairment in the activity of site I. The former possibility seems improbable, since the carriers for pyruvate and malate are different [32] and it would therefore be necessary to postulate that both carriers were affected. On the other hand, the fact that no alterations were observed with succinate as substrate, together with the normal response to ADP, suggests an efficient electron-transfer chain function. These data indicate that the decreased respiration with pyruvate-malate is probably due to a possible specific failure of site I in the cirrhotic rats. This interpretation is supported by the observation that neither the cytochrome content nor the ATPase activity was affected in submitochondrial particles from the experimental animals.

Oscillations of mitochondrial volume have been related to several phenomena, including proton and ion diffusion, energy-linked ion translocation and membrane structural modifications reflecting changes in its molecular conformation [15]. Changes in any of these parameters could be involved in the alterations of mitochondrial oscillation observed in the cirrhotic rats. However, since succinate was used as substrate and, as discussed above, the electron-transfer chain and oxidative phosphorylation seem to be unaffected, it is probable that membrane modifications may be responsible for the decreased oscillation parameters. The results of the determination of the acyl groups support this possibility, since a notable decrease in the mitochondrial unsaturated fatty acids was observed in the cirrhotic rats, without any change in the phospholipid pattern. In fact, it is known that mitochondria depleted of unsaturated fatty acids display an abnormal oscillatory behavior [33], probably because this modification of the acyl hydrophobic moiety results in a diminished fluidity of the mitochondrial membrane with a consequent limitation in the function of ion carriers.

The neurological symptoms of HE have been generally ascribed to extracerebral factors appearing as a consequence of liver failure, such as hyperammonemia, increased plasma levels of short-chain fatty acids, increased levels of mercaptans in blood and cerebrospinal fluid, and the presence of false neurotransmitters as octopamine [34]. Brain disfunction, including the structural alterations of glial cells observed in HE [35], could be the results of the effect of these factors.

At the present time it is difficult to suggest a mechanism for the alterations of brain mitochondrial function observed in this study. Although the possibility exists that CCl_4 may have produced mitochondrial damage through a direct toxic action on the brain, this seems improbable in light of the present knowledge of its mechanism of action. In fact, it has been established that CCl_4 produces liver damage by lipid peroxidation consequent to the formation of free radicals, mainly CCl_3, and in turn this reaction is greatly dependent on the activity of cytochrome P-450 located in the smooth endoplasmic reticulum [36–39]. Therefore, the sensitivity of the tissues to damage by CCl_4 and other toxic compounds acting through this mechanism is linked to their cytochrome P-450 content [36, 37, 40]. The brain possesses a very small concentration of this cytochrome as compared to that in the liver [37, 40], and it is practically insensitive to CCl_4 as judged by its covalent binding and lipid peroxidation stimulation [37]. In view of these data, one must conclude that

the alterations of brain mitochondria observed in the present study are probably the result of either the action of one or more of the extracerebral factors mentioned above or the consequence of other unidentified metabolic derangements. It is clear, however, that studies on the central nervous system must be carried out using other acute and chronic models of HE before a definitive conclusion can be reached.

It is also difficult to relate our findings to the pathogenesis and development of HE. All that can be said in this respect is that HE must be considered as a multifactorial syndrome, whose biochemical mechanism cannot be ascribed to a single specific alteration at a subcellular level in the brain.

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