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Cerebral Cortex Synaptic Heavy Mitochondria May Represent the Oldest Synaptic Mitochondrial Population: Biochemical Heterogeneity and Effects of L-Acetylcarnitine

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The microheterogeneous nature of intrasynaptic mitochondria has been demonstrated and is widely accepted. However, evidence is still lacking about the role played by the different intrasynaptic mitochondrial subpopulations. The data obtained support the hypothesis that "heavy" mitochondria could represent old mitochondrial populations: in fact, in addition to the well known impairment of typical mitochondrial functions, they possess the highest levels of hydroperoxides and their fatty acids pattern is completely modified. The qualitative and quantitative fatty acid modifications suffered by these organelles deeply altered their protein/lipid ratio, thus modifying their mode of action. The present work also collects a large body of evidence that a subchronic L-acetylcarnitine treatment in 28 days does not structurally affect both nonsynaptic and intrasynaptic mitochondria of normal rat in a "steady-state" metabolic condition.

KEY WORDS: Ubiquinone; synaptic and nonsynaptic mitochondria; lipid peroxidation; cytochromes; fatty acids

INTRODUCTION

In 1977, a method was developed whereby three distinct populations of relatively purified mitochondria, heterogeneous with respect to their biochemical features, could be prepared from a pool of rat forebrains (Lai *et al.*, 1977): two of them were derived from synaptosomes and a third consisted of "free" nonsynaptic mitochondria. Since then, increasing evidence for the heterogeneity of cerebral tissue as well as of cerebral mitochondria has been gathered in many morphological, histochemical, biochemical, and pharmacological studies (Hansford, 1983; Villa *et al.*, 1989a,b; Villa and Gorini, 1991a,b). Recently, heterogeneity at subcellular, cellular, and cerebral tissue regional levels was confirmed regarding enzymic activities (such as, monoamine oxidase and phosphorylase kinase) (Lai *et al.*, 1994; Psarra and Sotiroudis, 1996), preferential localization of active mitochondria in precursor cells (Kirischuk *et al.*, 1995), and different glutamate (Gorini *et al.*, 1998) and glutamine metabolism compartmentalization (Westergaard *et al.*, 1995). Our data previously contributed to support the hypothesis of the above-mentioned different levels of heterogeneity. We demonstrated, for example, that coenzyme Q distribution in brain mitochondria differs considerably depending on mitochondrial subpopulation, brain

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region, and age (Battino *et al.*, 1990, 1991, 1995, 1997). The respiratory chain features were also dependent on mitochondrial type and on their respective brain area (Villa *et al.*, 1989a; Villa and Gorini, 1991a; Battino *et al.*, 1991). Most of the studies dealing with nonsynaptic, "free" (FM) and intrasynaptic "light" (LM) and "heavy"(HM) mitochondria pointed out that the latter usually displayed the lowest content of respiratory components and the lowest enzymic activities both in the respiratory chain and in the matrix (Vanella *et al.*, 1989; Villa *et al.*, 1989a; Battino *et al.*, 1991). Because of these foregoing statements, the microheterogeneous nature of intrasynaptic mitochondria has been widely demonstrated and it has been suggested that HM fractions ("heavy" because of the different protein/lipid ratio) represent old mitochondrial populations at the end of their life. The continuous damage accumulating at membrane level could be responsible for this, leading to loss of enzymic activities and impairment of the typical mitochondrial function (Villa *et al.*, 1989a,b; Villa and Gorini, 1991a; Battino *et al.*, 1990, 1991, 1995, 1997). However, more evidence is still necessary to strengthen this hypothesis. On the other hand, carnitine and/or its esters are the requisite carriers for most of the transmembrane movements of long-chain fatty acids. Biochemically, L-acetylcarnitine (LAC) is involved in the transport of fatty acids across the mitochondrial membrane, regulating the cellular metabolism and increasing energy production (Shug *et al.*, 1982). LAC administration is associated with a reduction in some of the aging-related morphological changes and lipopigment deposition. This compound is a candidate for therapy as a long-term prophylactic agent for the adverse effects of cerebral aging (Dowson *et al.*, 1992). The effect of acute *in vivo* treatment with LAC was studied in different cerebral mitochondria from hippocampus and striatum during aging (4–24 months lifespan) showing its interference with some Krebs' cycle enzymes and glutamate metabolism (Villa and Gorini, 1991b). Moreover, LAC treatment increased the activity of cytochrome oxidase, indicating the specificity of molecular interactions with the catalytic activity of this enzyme during aging (Villa and Gorini, 1991b). This data was recently confirmed (Gorini *et al.*, 1998) and is consistent with previous studies (Turpeenoja *et al.*, 1988; Villa *et al.*, 1988) showing that LAC increased the amount of a 16kDa mitochondrial inner membrane protein, identified as the subunit IV of cytochrome oxidase (Ragusa *et al.*, 1989). This effect was also confirmed in humans (Corbucci *et al.*, 1992). Recently, it has been demonstrated (Gorini *et al.*, 1998) that LAC treatment has direct effects *in vivo* on several mitochondrial enzymic activities and can produce significant modification of their maximum rate (V_{max}). It has been found that such effects take place only in the intrasynaptic mitochondria.

Taking into account all these considerations, we decided to take advantage of our previous experiences concerning both LAC treatment and mitochondrial subpopulation separation to follow the possible effects of 28 days LAC administration on mitochondrial structure at the biochemical level; the aim of the present work was also to better structurally characterize HM fraction for supporting or not the hypothesis that they could represent the aged mitochondrial population, i.e., those mitochondria that were initially transported by axoplasmic flow.

EXPERIMENTAL PROCEDURE

Animal Care and Treatments

The experiments were performed on female Sprague-Dawley rats (Cobs-Charles River), aged 3 months. The animals were selected randomly and maintained under standard cycling and caging conditions (temperature, 22 ± 1°C; relative humidity, 60 ± 3%; lighting cycle, 12 h light and 12 h darkness; low noise disturbance), fed with a standard pellet diet, water *ad libitum*, and housed two per cage.

The time course of pharmacological treatment was established by permutation tables and the animals were treated for 28 days, 5 days a week, with intraperitoneal injections of: (i) group 1, vehicle only ($NaHCO_3$ 0.8 M; control animal); (ii) group 2, a dose of 30 mg/kg of L-acetylcarnitine; and (iii) group 3, a dose of 60 mg/kg of L-acetylcarnitine. The substance was injected each day at 10.00 A.M.. At a set time after the treatment cycle and after a wash-out of 48 hours, animals were sacrificed at 9.30 A.M. to avoid any circadian changes of enzyme activities. The animals were 4 months old when sacrificed.

Preparation of Synaptosomal Fraction and Nonsynaptic Mitochondria

A synaptosomal fraction was isolated according to the procedure of Lai *et al.* (1977) adapted for analytical evaluations (Villa *et al.*, 1989b). The rats, from

the various experimental lots, were anesthetized and sacrificed by urethane (1.4 g/kg, i.p.) and subsequent procedures were performed at 0–4°C. The brain was isolated ($<$20 s) in a refrigerated box at 0–4°C and immediately placed in the isolation medium (0.32 M sucrose, 1.0 mM EDTA-K^+, 10 mM Tris-HCI; pH 7.4). The left-side of the cerebral cortex was carefully dissected, isolated, and immediately placed in the isolation medium. The homogenate was obtained by Teflon glass homogenizer (Braun S Homogenizer) by five up-and-down passes of pestle (total clearance: 0.1 mm), rotating at 800 rpm (electronic control of the pestle speed). The homogenate was then centrifuged at 3.6×10^3 *g·min* in Beckman J2-21 Supercentrifuge, rotor JA-17. The pellet was resuspended in one-half original volume of isolation medium and again centrifuged at 3.6×10^3 *g·min*; this step was repeated once again. The three supernatants were combined and centrifuged at 288×10^3 *g·min*. The “crude” mitochondrial pellet containing synaptosomes was resuspended by soft homogenization in the isolation medium and was placed on discontinuous Ficoll–sucrose gradients (12–7.5% w/w) (Ficoll dissolved in stock solution: 0.32 M sucrose, 50 μM EDTA-K^+, 10 mM Tris-HCl, pH 7.4). The gradient was then centrifuged at 175.2 $\times 10^4 \times$ *g·min* in OTD-65B Sorvall Ultracentrifuge (AH-650 type rotor). The myelin fraction was collected by aspiration off and the synaptosomal band at 7.5–12% w/w Ficoll interphase was collected by aspiration, diluted threefold with isolation medium, and centrifuged at 288×10^3 *g·min*. The purified “free” mitochondrial pellet was resuspended in buffered sucrose 0.32 M, pH 7.4, and pelleted at 162.4×10^3 *g·min*. The pellet was resuspended in the same solution.

Preparation of Intrasynaptic Mitochondria

The synaptosomal pellet (previously isolated from Ficoll–sucrose gradient) was lysed by resuspension in 6 mM Tris-HCl, pH 8.1 medium, by soft homogenization (Villa *et al.*, 1989b). The lysate was then centrifuged at 399×10^3 *g·min* and the pellet was again resuspended and centrifuged at 192.6×10^3 *g·min*. The pellet was resuspended in a medium (3% w/w Ficoll, 0.12 M mannitol, 30 mM sucrose, 25 μM EDTA-K^+, 5 mM Tris-HCl, pH 7.4). This suspension was layered on a Ficoll discontinuous gradient consisting of two layers of 4.5% w/w Ficoll in 0.24 M mannitol, 60 mM sucrose, 50 μM EDTA-K^+, 10 mM Tris-HCl, pH 7.4 and, at the bottom, of 6% w/w Ficoll in the same solution. This gradient was centrifuged at 280.2×10^3 *g·min*. At the end of this centrifugation, the upper phase (“light” intrasynaptic mitochondrial fraction) was aspirated and pelleted at 166.5×10^3 *g·min*. The pellet of this centrifugation and that from the gradient (“heavy” intrasynaptic mitochondrial fraction) were separately resuspended in 0.32 M sucrose-buffered solution (pH 7.4) and centrifuged at 162.4×10^3 *g·min*. The washed pellets were finally resuspended in the same washing solution.

Biochemical Assays

Cytochrome Contents

The cytochrome contents were evaluated by the differential spectra (dithionite reduced minus ferricyanide oxidized in the presence of 1% deoxycholate) in a Beckman DU-640 spectrophotometer according to Vanneste (1966) and as previously indicated (Battino *et al.*, 1991).

Coenzyme Q_9 and Q_{10} (CoQ_9, CoQ_{10}) Contents

After extraction with methanol and light petroleum, according to the method of Kröger (1978), mitochondria were assayed for CoQ contents by reversed-phase HPLC analysis as previously reported (Battino *et al.*, 1990, 1991, 1995) in a Beckman System Gold equipped with a Diode Array detector and performing separations at 25°C using a Spherisorb S5 ODS I column.

Hydroperoxides

The ferrous oxide xylenol orange (FOX2) method was used for determining hydroperoxides (HP). HP levels were assayed according to the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions (Jiang *et al.*, 1992), slightly modified (Nouroz-Zadeh *et al.*, 1994), using tryphenylphosphine (TPP), an agent that avoids artifactual color generation in samples that might contain substantial quantities of loosely available iron. Briefly, mitochondria (0.1 mg) were incubated at 37°C for 30 min with and without 1 mM TPP. FOX2 reagent was then added to each sample and incubated again at 37°C for 30 min in a water shaking bath. After centrifugation

(2000 × *g* for 5 min) the supernatants were monitored at 560 nm. Moreover, the samples were also challenged, *in vitro*, by further peroxidative attack, in order to elucidate the maximal levels of peroxidative modification they can undergo. This was attained by incubating mitochondria at 37°C for 30 min in the presence of 2 mM 2,2′-azobis(amidinopropane hydrochloride) (AAPH), a chemical free-radical initiator.

Fatty Acid Composition

Mitochondrial fatty acid composition was determined, as described by Lepage and Roy (1986), after transesterification in a Hewlett Packard HP 5890 Series II chromatograph, using a 60-m long capillary column, 32-mm id and 20-mm thickness impregnated with Spy 2330 FS (Supelco Inc. Bellefonte, Palo Alto, CA, USA). Mitochondrial fatty acids patterns were calculated on a percentage basis as well as the relative contents of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), or the unsaturation index (UI).

Protein Determination

The protein content of samples was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Phosphorus Determination

Phospholipid phosphorus was determined using the method of Marinetti (1962).

Statistical Analysis

The ANOVA test was used to evaluate interactions between control and LAC-treated animals, for each mitochondrial fraction, and for each biochemical parameter tested. Homogeneity of variance was checked by Levene's test. When significant differences were detected ($p < 0.05$), Scheffè's test was used for a post hoc comparison. All tests were performed using a PC Statistical Package (SPSS).

RESULTS

As in the original work for the subfractionation of these mitochondria from hippocampus (Villa *et al.*, 1989b), the purity of these mitochondria obtained from cerebral cortex was assessed by the evaluation of lactate dehydrogenase (Bergmeyer and Bernt, 1974), as a cytoplasmic (or synaptoplasmic) marker (Johnson and Whittaker, 1963), and acetylcholinesterase (Ellman *et al.*, 1961), as a synaptic plasma membrane and membrane material marker enzyme (Cotman and Matthews, 1971). *Actual high-purity mitochondria should display low activity of the marker enzymes*. The lactate dehydrogenase activity here was equal to 0.07 and 0.1% of homogenate for "light" and "heavy" mitochondria, respectively; acetylcholinesterase activity was equal to 0.4% for both "light" and "heavy" mitochondria, confirming that the purity of these mitochondria was the same for both "light" and "heavy" ones obtained from the hippocampus (Villa *et al.*, 1989b) and comparable to values reported previously by us, obtained from frontal cerebral cortex of rat brain (Vanella *et al.*, 1989).

The cytochrome concentrations were investigated by their differential spectra only in nonsynaptic mitochondria because the intrasynaptic mitochondrial fractions were quantitatively insufficient for these determinations. The shape of the absorbance spectra obtained is typical for nonsynaptic mitochondria: in fact, cytochrome $c + c_1$, b and $a + a_3$ revealed net and sharp absorbance maxima at 550, 561, and 605 nm, respectively. LAC treatment did not affect cytochrome contents at both LAC concentrations used. Figure 1 shows that cytochrome $c + c_1$, b, and $a + a_3$ levels of FM mitochondria did not significantly change after 28 days drug administration.

The limited amount of samples available made it possible to investigate the CoQ_n contents of FM only. Total mitochondrial CoQ (*i.e.*, $CoQ_9 + CoQ_{10}$), was not affected by LAC treatment (Fig. 2); however, although it is not significant, a lower CoQ amount was found with a LAC dose of 30 mg/kg and such a pattern is similar to the one found for CoQ_9 and CoQ_{10} contents (Fig. 2). While the former is still not significant, the latter is ($p < 0.05$) and the CoQ_{10}/CoQ_9 ratio did not change (data not shown) as its value was always between 0.53 and 0.55.

HM had the highest HP contents (Fig. 3a), about 30% more than FM and LM ($p < 0.05$); HP in LM were higher than in FM, but the differences were limited. LAC treatment did not induce changes of the

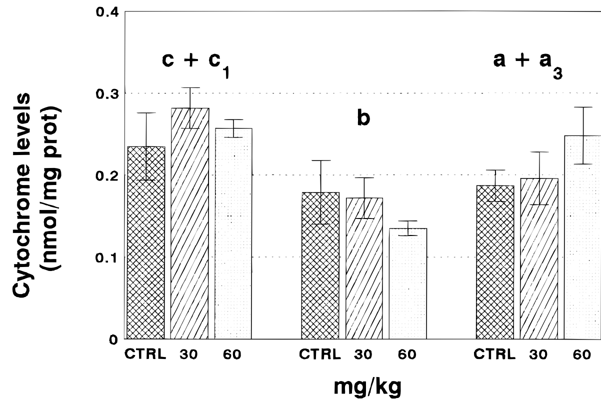

Fig. 1. Cytochrome $c + c_1$, b, and $a + a_3$ concentrations expressed in nmoles × mg (of mitochondrial protein)$^{-1}$ as assayed on the nonsynaptic "Free" (FM) mitochondrial fraction from left-side cerebral cortex of Sprague-Dawley rats aged 4 months: Control (vehicle: CTRL) and treated by L-acetylcarnitine at the dose of 30 and 60 mg/kg/day body wt for 28 days. Values represented as means ± SEM (n = 11). The statistical analysis was performed as indicated in the text.

basal concentration of HP in any of the three subpopulations of mitochondria. The extent of AAPH-induced peroxidation differed among the different kinds of mitochondria with the highest values found in FM and the lowest ones in HM ($p < 0.05$ for FM versus LM, FM versus HM, and LM versus HM) (Fig. 3b). In fact, FM increased from about 0.09 to about 0.28 μmol/mg protein; values for LM were from about 0.1 to about 0.24 μmol/mg protein and, finally, HM changed from about 0.145 to about 0.2 μmol/mg protein. Again, LAC treatment did not have any influence on these

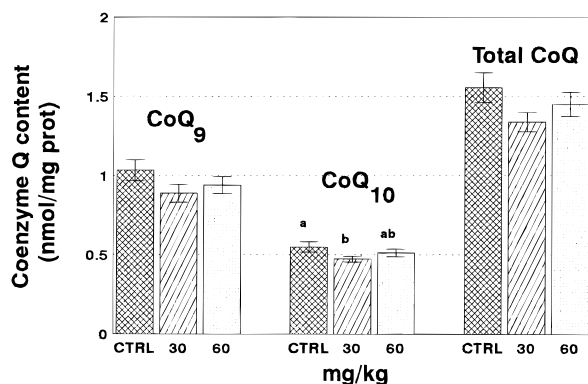

Fig. 2. Coenzyme Q_9, coenzyme Q_{10}, and total coenzyme Q concentrations expressed in nanomoles × milligram of mitochondrial protein^{-1} as assayed in the mitochondrial fraction indicated in Fig. 1. Values represented as means ± SEM (n = 11). Means with different superscripts are significantly different ($p < 0.05$). The statistical analysis was performed as indicated in the text.

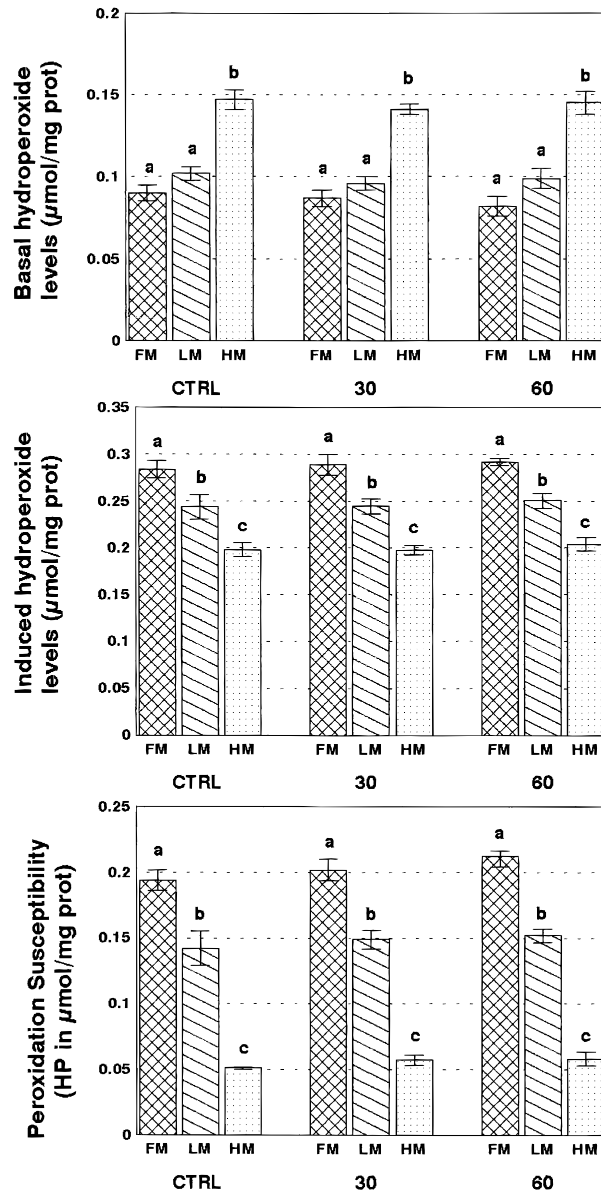

Fig. 3. (a) Native hydroperoxide concentrations expressed in micromoles × milligram of mitochondrial protein^{-1} as assayed on the nonsynaptic "Free" (FM), intrasynaptic "Light" (LM), and "Heavy" (HM) mitochondrial fractions from left-side cerebral cortex of 4-month-old Sprague-Dawley rats: control (vehicle: CTRL) and treated by L-acetylcarnitine at a dose of 30 and 60 mg/kg/day body wt for 28 days. Values represented as means ± SEM (n = 11). Means with different superscripts are significantly different ($p < 0.05$). The statistical analysis was performed as indicated in the text. (b) Hydroperoxide levels after AAPH-induced peroxidation. Samples and abbreviations as in (a). (c) Evaluation of proneness to oxidation of the different mitochondrial populations, *i.e.*, amount of hydroperoxide that depends on the *in vitro* oxidation; samples and abbreviations as in (a).

biochemical parameters. The effects produced by AAPH are even more evident in Fig. 3c, where the amount of HP due to the AAPH action is taken into account. The differences are more pronounced than in Fig. 3b: HM proneness to peroxidation was 30 and 25% of LM and FM, respectively ($p < 0.05$). In addition, LM evidenced a lower susceptibility to oxidation than FM ($p < 0.05$). Once again, LAC did not modify these parameters.

LAC administration, however, partially influenced HP/CoQ molar ratio: it appears that the pharmacological treatment had the tendency to increase the ratio at the dose of 30 mg/kg while the higher dose is ineffective (Fig. 4a). Such a tendency is confirmed when the peroxidation is stimulated with AAPH. In this situation, either the amount of induced HP or the evaluation of sample oxidizability (Fig. 4b,c, respectively) indicate that a LAC dose of 30 mg/kg significantly increased HP/CoQ molar ratio ($p < 0.05$ for control versus 30) while the effect elicited by 60 mg/kg was less evident and not statistically different.

Mitochondrial fatty acid profile (each fatty acid amount is expressed as percentage of total fatty acids) is shown in Table I. Once again, pharmacological treatment did not significantly alter the relative fatty acid content in each subpopulation. On the one hand, very significant differences were found among mitochondrial subpopulations, often irrespective of the pharmacological administration. The fatty acids that appeared to present the most drastic differences were 14:0, 18:0, 20:4n-6, 22:4n-6, 24:1n-9, 22:5n-6, and 22:6n-3 when FM and LM were compared with HM. In percentage, HM contained six times more 22:5n-6 and 22-6n-3 than FM and LM, three times more 14:0, 22:4n-6, and 24:1n-9, two times more 20:4, 20% more 16:0 and 22:5n-3, and, finally, 25 and 65% less 18:1 and 18:0, respectively. On the other hand, 16:1, 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:3n-6, 20:5n-3, and 24:0 completely lacked in HM patterns. Table II shows that similar indications are evident also from the most common and useful indexes in fatty acid composition analysis. In fact, SFA, UFA, MUFA:PUFA, PUFA(n-6), PUFA(n-3), UI, SFA/UFA(n-6), and 18:1/18:0 differed significantly in FM, or LM, with respect to HM. The fatty acid analysis was always performed on the basis of the same sample amount (*i.e.*, 100 μg mitochondrial protein). However, dramatic qualitative, as well as quantitative, differences were found in fatty acid patterns between the mitochondrial subpopulations. The total fatty acid amount in HM was about 20% of that assayed in the corresponding FM, or LM,

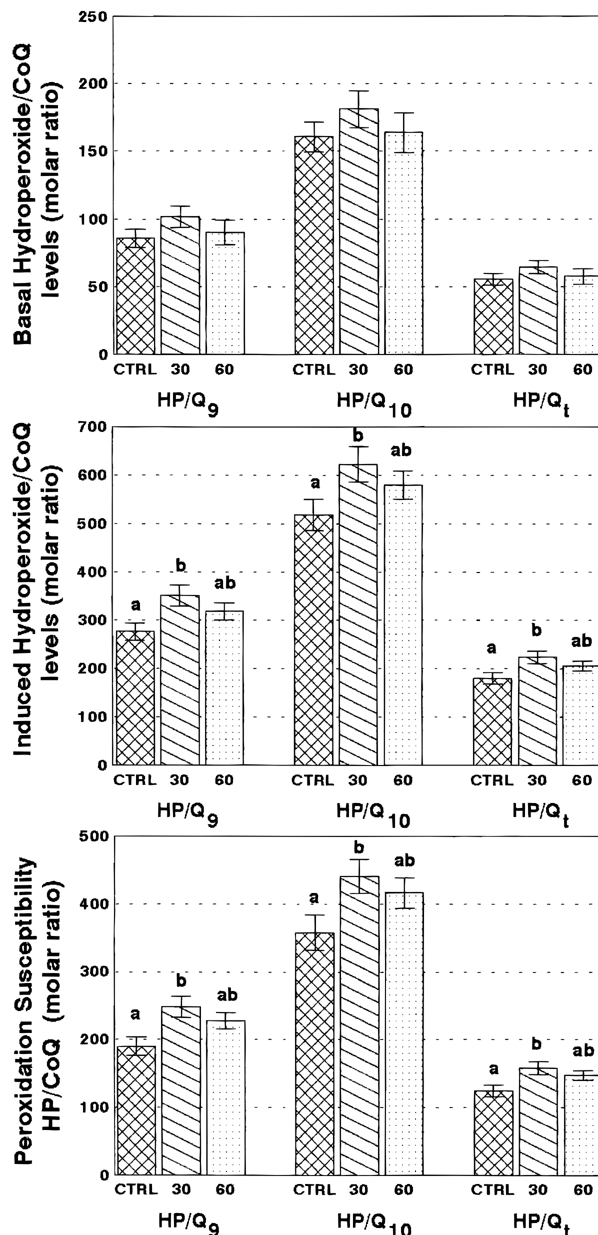

Fig. 4. (a) Native hydroperoxide/coenzyme Q ratios as calculated for the nonsynaptic "Free" (FM) mitochondrial fraction from left-side cerebral cortex of 4-month-old Sprague-Dawley rats: control (vehicle: CTRL) and treated by L-acetylcarnitine at a dose of 30 and 60 mg/kg/day body wt for 28 days. Values represented as means ± SEM ($n = 11$). Means with different superscripts are significantly different ($p < 0.05$). The statistical analysis was performed as indicated in the text. (b) Hydroperoxide/CoQ ratios after AAPH-induced peroxidation. Samples and abbreviations as in (a). (c) Evaluation of proneness to oxidation of the FM fraction considering the *in vitro* peroxidation-induced changes in the hydroperoxide/CoQ ratios; samples and abbreviations as in (a).

Table I. Mitochondrial Fatty Acid Patterns[a]

	FM Cont ($n = 10$) a	FM 30 ($n = 11$) b	FM 60 ($n = 8$) c	LM Cont ($n = 6$) d	LM 30 ($n = 5$) e	LM 60 ($n = 6$) f	HM Cont ($n = 7$) g	HM 30 ($n = 7$) h	HM 60 ($n = 8$) i	Statistical analysis ($p < 0.05$)
14:0	2.2 ± 0.05	1.96 ± 0.07	2.33 ± 0.07	2.51 ± 0.32	2.28 ± 0.1	2.17 ± 0.06	7.67 ± 2.47	6.06 ± 2.1	4.21 ± 1.4	—
16:0	35 ± 0.25	34.98 ± 0.25	35.18 ± 0.38	38.37 ± 0.6	37.35 ± 1.54	36.72 ± 0.69	46.43 ± 6.28	44.23 ± 6.16	41.31 ± 6.95	—
16:1	1.93 ± 0.15	1.59 ± 0.14	2.05 ± 0.13	2.38 ± 0.15	2.3 ± 0.15	2.13 ± 0.17	n.d.[b]	n.d.	n.d.	—
18:0	34.43 ± 0.29	34.33 ± 0.22	33.73 ± 0.4	29.42 ± 1.22	29.04 ± 0.87	29.63 ± 1.5	10.16 ± 2.09	10.85 ± 1.71	10.32 ± 1.56	g vs. a,d/b vs. e,h; e vs. h/i vs. c,f
18:1	11.41 ± 0.09	11.68 ± 0.17	11.12 ± 0.16	10.41 ± 0.5	11.18 ± 0.29	11.27 ± 0.45	8.6 ± 1.39	10.38 ± 1.7	9.64 ± 1.74	—
18:2n-6	2.05 ± 0.08	2.06 ± 0.07	2.09 ± 0.06	1.99 ± 0.1	2.08 ± 0.12	2.05 ± 0.03	n.d.	n.d.	n.d.	—
18:3n-6	0.76 ± 0.04	0.78 ± 0.06	0.79 ± 0.04	0.96 ± 0.05	1.13 ± 0.09	0.91 ± 0.07	n.d.	n.d.	n.d.	b vs. e
18:3n-3	0.72 ± 0.08	0.79 ± 0.11	0.66 ± 0.06	0.98 ± 0.11	0.94 ± 0.08	1.24 ± 0.17	n.d.	n.d.	n.d.	b vs. e/c vs. f
20:2n-6	0.53 ± 0.04	0.59 ± 0.04	0.59 ± 0.05	0.94 ± 0.1	1.02 ± 0.17	1.04 ± 0.13	n.d.	n.d.	n.d.	a vs. d/b vs. e/c vs. f
20:3n-6	0.74 ± 0.08	0.68 ± 0.05	0.73 ± 0.07	0.76 ± 0.11	0.7 ± 0.11	0.76 ± 0.07	n.d.	n.d.	n.d.	—
20:4n-6	1.66 ± 0.06	1.66 ± 0.09	1.77 ± 0.08	1.82 ± 0.22	2.06 ± 0.28	1.91 ± 0.29	3.18 ± 0.51	4.11 ± 0.65	3.63 ± 0.72	g vs. a,d/h vs. b,e/i vs. c,f
20:5n-3	0.87 ± 0.04	0.92 ± 0.05	0.96 ± 0.06	1.76 ± 0.16	1.86 ± 0.25	1.58 ± 0.28	n.d.	n.d.	n.d.	a vs. d/b vs. e
22:4n-6	1.67 ± 0.07	1.79 ± 0.06	1.89 ± 0.11	1.69 ± 0.15	1.78 ± 0.2	1.97 ± 0.27	4.55 ± 0.82	4.97 ± 0.81	5.41 ± 0.77	g vs. a,d/h vs. b,e/i vs. c,f
24:0	0.92 ± 0.06	1.1 ± 0.1	0.97 ± 0.11	1.22 ± 0.13	1.4 ± 0.22	1.4 ± 0.23	n.d.	n.d.	n.d.	—
24:1n-9	0.89 ± 0.07	0.83 ± 0.05	0.84 ± 0.05	1.01 ± 0.17	1.01 ± 0.07	1.02 ± 0.12	2.58 ± 0.6	2.74 ± 0.51	4.67 ± 1.05	g vs. a,d/h vs. b,e/i vs. c,f
22:5n-6	1.12 ± 0.07	1.25 ± 0.11	1.11 ± 0.08	1.00 ± 0.3	1.01 ± 0.14	1.09 ± 0.2	6.97 ± 1.59	7.21 ± 1.26	9.73 ± 1.69	g vs. a,d/h vs. b,e/i vs. c,f
22:5n-3	1.75 ± 0.06	1.74 ± 0.08	1.73 ± 0.09	1.62 ± 0.13	1.76 ± 0.12	1.9 ± 0.19	2.16 ± 0.45	2.22 ± 0.36	3.9 ± 0.64	e vs. h/i vs. c
22:6n-3	1.34 ± 0.15	1.28 ± 0.13	1.46 ± 0.16	1.19 ± 0.1	1.09 ± 0.15	1.2 ± 0.21	7.69 ± 1.88	7.22 ± 1.38	7.18 ± 1.43	g vs. a,d/h vs. b,e/i vs. c,f

[a] Fatty acid amount is expressed in percentage of total fatty acids. Data are means ± SEM; $n = 11$.
[b] n.d., not detectable.

Table II. Parameters Obtained from the Mitochondrial Fatty Acid Patterns[a]

	FM Cont (n = 10) d	FM 30 (n = 11) b	FM 60 (n = 8) c	LM Cont (n = 6) d	LM 30 (n = 5) e	LM 60 (n = 6) f	HM Cont (n = 7) g	HM 30 (n = 7) h	HM 60 (n = 8) i	Statistical analysis ($p < 0.05$)
SFA	72.55 ± 0.44	72.37 ± 0.35	72.21 ± 0.39	71.52 ± 0.93	70.07 ± 1.74	69.93 ± 1.67	64.26 ± 6.73	61.15 ± 6.46	55.84 ± 6.84	h vs. b,e/i vs. c,f
UFA	27.45 ± 0.44	27.63 ± 0.35	27.79 ± 0.39	28.48 ± 0.93	29.93 ± 1.74	30.07 ± 1.67	35.74 ± 6.73	38.85 ± 6.46	44.16 ± 6.84	h vs. b,e/i vs. c,f
MUFA	14.23 ± 0.17	14.09 ± 0.25	14.02 ± 0.19	13.79 ± 0.51	14.49 ± 0.38	14.42 ± 0.47	11.18 ± 1.91	13.12 ± 2.14	14.3 ± 2.27	—
DUFA	2.59 ± 0.09	2.65 ± 0.08	2.68 ± 0.05	2.93 ± 0.13	3.1 ± 0.26	3.1 ± 0.15				—
MUFA:DUFA	5.55 ± 0.16	5.34 ± 0.08	5.24 ± 0.1	4.77 ± 0.29	4.79 ± 0.34	4.71 ± 0.26				—
MUFA:PUFA	1.08 ± 0.02	1.05 ± 0.03	1.03 ± 0.05	0.97 ± 0.09	0.97 ± 0.08	0.98 ± 0.11	0.48 ± 0.02	0.52 ± 0.02	0.48 ± 0.01	g vs. a,d/h vs. b,e/i vs. c,f
PUFA(n-6)	8.54 ± 0.21	8.8 ± 0.2	8.98 ± 0.23	9.15 ± 0.69	9.78 ± 0.96	9.72 ± 0.96	14.7 ± 2.74	16.29 ± 2.67	18.77 ± 2.91	g vs. a/h vs. b,e/i vs. c,f
PUFA(n-3)	4.68 ± 0.2	4.73 ± 0.17	4.8 ± 0.29	5.54 ± 0.43	5.66 ± 0.5	5.93 ± 0.67	9.86 ± 2.32	9.44 ± 1.73	11.08 ± 1.74	g vs. a/h vs. b,e/i vs. c,f
PUFA > 18C(n-6)	5.73 ± 0.13	5.97 ± 0.19	6.09 ± 0.24	6.2 ± 0.71	6.56 ± 0.79	6.76 ± 0.92				—
PUFA > 18C(n-3)	3.96 ± 0.2	3.94 ± 0.18	4.14 ± 0.25	4.56 ± 0.33	4.72 ± 0.51	4.69 ± 0.59				—
UI	0.91 ± 0.02	0.93 ± 0.02	0.95 ± 0.03	0.99 ± 0.07	1.07 ± 0.11	1.09 ± 0.13	88.65 ± 16.5	97.45 ± 15.9	119.6 ± 17.5	g vs. a,d/h vs. b,e/i vs. c,f
SFA/MUFA	2.65 ± 0.06	2.63 ± 0.05	2.6 ± 0.05	2.53 ± 0.11	2.39 ± 0.2	2.38 ± 0.19	2.58 ± 0.71	2.17 ± 0.63	1.7 ± 0.41	h vs. b,e/i vs. c
20:4/18:2	0.85 ± 0.06	0.79 ± 0.05	0.85 ± 0.03	0.94 ± 0.15	0.97 ± 0.1	0.93 ± 0.13				—
18:1/18:0	0.33 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	0.39 ± 0.02	0.38 ± 0.02	0.88 ±0.05	0.95 ± 0.02	0.91 ± 0.04	g vs. a,d/h vs. b,e/i vs. c,f

[a] Fatty acid amount is expressed in percentage of total fatty acids.
Data are means ± SEM; n = 11.

subpopulations. In fact, if the quantitative value of control FM fraction is taken as a reference value, the HM fractions, regardless of the pharmacological treatment, displayed decreases of 75% or more. The analysis of phospholipid phosphorus revealed that no differences in phosphorus content were present in any of the mitochondrial groups assayed (data not shown).

DISCUSSION

Most of the studies on brain mitochondria have been carried out using pooled whole brain hemispheres or cortex. However, the brain is made up of heterogeneous tissue variously distributed in different zones with distinct metabolic activities responsible for diverse functions (Villa *et al.*, 1989a). Even more significance should be given to these considerations when subcellular investigations are made (Villa *et al.*, 1989a, b; Battino *et al.*, 1991, 1995, 1997). Evidence was found supporting a biochemical diversity between perikaryal and intrasynaptic mitochondria, although the nature and significance of the differences were not clear, especially with regard to diversity between intrasynaptic mitochondria (Vanella *et al.*, 1989). Specific attention was devoted to the microheterogeneity of intrasynaptic mitochondria. The results of previous investigations (*i.e.*, loss of antioxidant molecules, decrease in several enzymic activities) suggest that HM may represent an aged mitochondrial population characterized by a partial impairment of typical mitochondrial function (Battino *et al.*, 1991, 1995, 1997).

The results of the present work may sustain the above-mentioned hypothesis. In fact, HM actually showed the higher content of native HP. In other words, they appeared to be the most oxidatively prejudiced among the subpopulations studied (Fig. 3a). The lowest level of oxidative damage was found in FM, but the relative higher contents in LM was never significant. Such limited differences between FM and LM are in accordance with previous indications on either respiratory chain activities (succinate: cytochrome *c* oxidoreductase, succinate dehydrogenase, ubiquinol: cytochrome *c* oxidoreductase, and cytochrome oxidase) or CoQ_9 CoQ_{10} contents (Battino *et al.*, 1991). The fact that HM are the most damaged mitochondria could fit perfectly with the very low enzymic activities and low antioxidant contents typical of this subpopulation. The data of Figs. 3b, c confirm further the above indications. The three mitochondrial subpopulations were exposed *in vitro* to the action of a free-radical initiator in order to elicit the maximum possible peroxidative damage to their structure. Results from this challenge indicate (Fig. 3b) that three statistically different extents of peroxidation were reached. FM reached a 200% increase in relation to HP basal levels, LM increments were lower but still remarkable (about 150%), and, finally, HM only increased by about 30%. The reasons for such behavior should be ascribed to two main facts: (1) the peroxidizable material (*i.e.*, PUFA) of HM populations is, in its majority, already naturally highly oxidized; (2) the lipid/protein ratio is lower in HM than in LM and FM. Point (2) is partially an effect of what occurred in (1): it is reasonable that at least a part of the oxidized and damaged fatty acids are eliminated by the appropriate cellular devices, especially in young rats. However, the efficiency of these mechanisms may be limited with age and the large extent of modifications occurring in HM at a membrane level cannot be completely repaired in this way, thus producing an accumulation of injuries. A highly oxidized, functionally jeopardized, physically heavy organelle could be the final result of the progressive augmentation of noxious stimuli during the lifespan of synaptic mitochondria that we usually isolate as HM.

Fatty acid analysis could also support this hypothesis. The mere comparison of gas chromatograms obtained from HM and LM (or FM) showed that something dramatic had occurred in HM. From a quantitative point of view, about 75% of the total amount of fatty acids were lacking in HM profile. Moreover, the pattern of the remaining fatty acids completely differed from LM (and FM) from a qualitative point of view. An analysis of phosphorus content per mitochondrial protein did not reveal any variation among the mitochondrial fractions. This indicates that part of the fatty acids were taken away from their respective phospholipids or that HM membranes had, in some way, “lost” part of their fatty acids. This is, indeed, more evident when accounting for the altered lipid/protein ratio of HM respect to LM. In addition, the remaining few fatty acids are saturated or highly polyunsaturated (Table I). Fatty acids with a high level of polyunsaturation are highly oxidized (as demonstrated above) or have a high susceptibility to be completely oxidized. The MUFA/PUFA ratio (Table II), usually an index of the membrane susceptibility to peroxidation, was only half in HM compared to LM or FM. The correlation between the MUFA:PUFA index in membrane and the ability of the latter to withstand lipid peroxidation is supported by previous works on different conditions causing oxi-

dative stress (Huertas *et al.*, 1991, Mataix *et al*, 1998) and could even be more important than the net PUFA content itself. Several more indexes were also significantly modified (Table II), some of which in a dramatic manner, particularly UI, which was found to be three orders of magnitude greater in HM than LM. The ratio 18:1/18:0 and 20:4/18:2 indexes are product precursor ratios for the reactions controlled by Δ-9 desaturase, in the first case, and Δ-5 and Δ-6 desaturases, in the second, and are used as indexes for the activity of these enzymes. In the case of 18:1/18:0, this index underwent a threefold increment in HM, suggesting that these mitochondria are also affected by altered relationships with such cellular enzymes. The high proportion of PUFA in HM mitochondria, together with the modifications on the saturated fraction, could be both part of a higher regulation mechanism. In this sense, other authors (Periago *et al.*, 1990) and our group (Huertas *et al.* 1992; Quiles *et al.*, 1999) have previously described how mitochondrial membranes are able to modify their fatty acid profile to maintain a constant membrane fluidity. This hypothesis agrees with the substitution of saturated fatty acid in the membrane by PUFA, which is less rigid than the former. In the same way, this mechanism could be a consequence of the previously reported loss in enzymic activities in HM mitochondria because of the aging imbalance in this population. In summary, the mitochondria could modify the composition of their membrane in order to maintain functionality. However, these changes are not sufficient to counterbalance the accumulated damages; indeed, they contribute to the same by increasing the oxidizability of the membrane.

The cytochrome contents of FM fraction agree with previous data (Fig. 1). However, data indicate higher, although not significant, levels of all cytochromes in the present study. Once again, the high degree of brain heterogeneity probably accounts for them. The case of cytochromes in FM is emblematic e.g., cytochrome $c + c_1$ content is about 70% higher in striatum (or about 50% higher in hippocampus) than in right-side cerebral cortex FM (Battino *et al.*, 1991). The data of the present study were obtained from the analysis of left-side cerebral cortex mitochondria and revealed constant, moderately higher, cytochrome contents than FM from right-side cerebral cortex indicating that a macroheterogeneity also exists between cerebral functional areas (Villa *et al.*, 1989a).

As far as CoQ content in FM is concerned, the data (Fig. 2) reflect the same situation found in the case of cytochromes. Briefly, minor differences were found (an increment of about 12% for CoQ_9 and about 18% for CoQ_{10}) compared to results concerning right-side cerebral cortex (Battino *et al.*, 1991). However, the CoQ_{10}/CoQ_9 ratio was practically unaffected (between 0.53 and 0.55) by such minor differences and is identical to those calculated in previous studies for rats of the same age. It is worthwhile to consider that despite the variations due to brain heterogeneity, the CoQ_{10}/CoQ_9 ratio remains sufficiently constant regardless of the brain area and the mitochondrial fraction considered (Battino *et al.*, 1991, 1995, 1997). It seems that such constant ratio could be critical for minimal efficiency of the mitochondrial machinery (Battino, 2000).

The only parameters that appeared to be significantly affected by LAC administration were the HP/CoQ ratio and CoQ_{10} levels. A similar, but opposite trend (without statistical significance), was found both for CoQ_9 and basal HP/CoQ values. Partial modification of HP/CoQ ratio after pharmacological treatment has already been found (Battino *et al.*, 1996). The tendency for HP/CoQ ratio to increase (and concomitantly for CoQ_{10} to decrease) and subsequently recover the basal level might be interpreted as the consequence of the initial alteration of cellular steady-state function followed by induction phenomena tending to reestablish the primitive situation. This defensive device, along with other similarly activated systems, such as glutathione peroxidase (Scarpa *et al.*, 1987), is widespread in rat brain.

The present work collects a large body of evidence in the sense that a subchronic LAC treatment for 28 days does not structurally affect both nonsynaptic and intrasynaptic mitochondria of normal rat in steady-state metabolic condition because, probably, the influence of LAC resides only at a functional level, influencing some specific mitochondrial enzymatic activities (Gorini *et al.*, 1998). In addition, because the free mitochondrial fraction also contains mitochondria from glia (Villa *et al.*, 1989b) the higher susceptibility to peroxidative stress may be of importance during the developments of sequelae from noxious stimuli. In this sense, the possibility of having three mitochondrial populations from the same animals, may be a very interesting pharmacological model with which to evaluate the effects of drugs acting on peroxidative processes (Villa and Gorini, 1997).

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