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Structural Damage Induced by Peroxidation May Account for Functional Impairment of Heavy Synaptic Mitochondria

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Coenzyme Q distribution, as well as respiratory chain features, in rat brain mitochondria depend on mitochondrial subpopulation, brain region and age. Heavy mitochondria (HM) usually display the lowest content of respiratory components and the lowest enzymatic activities and it has been suggested that they represent the oldest mitochondrial population. In this study, we confirmed that HM are considerably compromised in their structure. In fact, HM showed to have the highest hydroperoxide content and the most consistent modifications in their fatty acid pattern with wide loss of fatty acids (or part of them) in the phospholipid moiety. Such situation could explain the typical impairment of HM and could support the hypothesis that they represent an old mitochondrial population.

Keywords: Coenzyme Q; Hydroperoxides; Aging; Peroxidation; Rat brain mitochondria

INTRODUCTION

Coenzyme Q (CoQ) distribution in rat brain mitochondria depends on mitochondrial subpopulation, brain region and age.[1–5] Similarly, the respiratory chain features also depend on mitochondrial type and on their respective brain area.[2,6,7] Such data are only a part of a large body of evidence, that has been accumulating since the '70,[8] demonstrating from morphological, histochemical, biochemical and pharmacological point of view that brain heterogeneity exists at subcellular, cellular and cerebral tissue regional levels.[6,7,9–12] Mitochondria probably represent the main expression of such heterogeneity. Several studies regarding non-synaptic "free" (FM), intra-synaptic "light" (LM) and "heavy" (HM) mitochondria indicated the latter ("heavy" because of the presumably different protein/lipid ratio) as having the lowest content of respiratory components and the lowest enzymatic activities both in the respiratory chain and in the matrix.[1–6,13] Due to this micro-heterogeneous nature of intra-synaptic mitochondria it has been suggested that HM fractions represent the oldest mitochondrial populations. The physiological, continuous damage accumulating at mitochondrial level as a result of both electron leakage at complex I site[14] as well as of superoxide anion production,[15] could be responsible for loss of enzymatic activities and impairment of the typical mitochondrial functions. The aim of the present work was to better characterize HM fraction in order to investigate whether or not it could represent the aged mitochondrial population, i.e. those mitochondria that were initially transported by axoplasmic flow

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and that, for this reason, have undergone a more protracted endogenous oxidative insults.

MATERIAL AND METHODS

The experiments were performed on female Sprague–Dawley rats (Cobs-Charles River) 4 months of age. The animals were selected randomly, kept from birth 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 animals were sacrificed at 9.30 a.m. to avoid any circadian changes of enzyme activities.

Preparation of Synaptosomal Fraction and Non-synaptic Mitochondria

The synaptosomal fraction was isolated according to Lai *et al.*[8] modified for analytical evaluations.[10] The rats, were anesthetized and sacrificed by urethane (1.4 g/kg, i.p.) and subsequent procedures were performed at 0–4°C. The brain was rapidly 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–HCl, pH 7.4). The left-side of the cerebral cortex was carefully dissected (i.e. eliminating the myelin in order to avoid any interference on the gradient), isolated and immediately placed in the isolation medium. The homogenate was obtained by employing a teflon glass homogenizer (Braun S Homogenizer). 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 the 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), 1.9 ml of each). The gradient was then centrifuged at 175.2 × $10^4 g$ min in OTD-65B Sorvall Ultracentrifuge (AH-650 type rotor). The myelin fraction was sucked off and the synaptosomal band at 7.5–12% (w/w) Ficoll interphase was collected by aspiration, diluted three-fold 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 pelletted at 162.4 × $10^3 g$ min. The pellet was resuspended in the same solution.

Preparation of Intra-synaptic Mitochondria

The synaptosomal pellet (previously isolated from Ficoll-sucrose gradient) was lysed by resuspension of 6 mM Tris–HCl, pH 8.1 medium, by soft homogenization.[10] The lysate was then centrifuged at 399 × $10^3 g$ min and the pellet was again resuspended and centrifuged at 192.6 × 103g min. The pellet was resuspended in a medium (3% (w/w) Ficoll, 0.12 M mannitol, 30 mM sucrose, 25 mM EDTA-K^+, 5 mM Tris–HCl, pH 7.4). This suspension was layered on Ficoll discontinuous gradient consisting of two layers, the first one consisting of 1.4 ml of 4.5% (w/w) Ficoll (in 0.24 M mannitol, 60 mM sucrose, 50 mM EDTA-K^+, 10 mM Tris–HCl, pH 7.4) and a second one at the bottom consisting of 2.6 ml of 6% (w/w) Ficoll. This gradient was centrifuged at 280.2 × $10^3 g$ min. At the end of this centrifugation, the upper phase ("light" intra-synaptic mitochondrial fraction) was sucked off and pelletted at 166.5 × $10^3 g$ min. The pellet of this centrifugation and that from the gradient ("heavy" intra-synaptic mitochodrial 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.

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

After extraction with methanol and light petroleum,[16] mitochondria were assayed for Coenzyme Q (CoQ) contents by reversed-phase HPLC analysis as previously reported[1–3] in a Beckman System Gold equipped with a Diode Array detector and performing separations at 25°C using a Spherisorb S5 ODSI 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[17] using tryphenylphosphine (TPP), an agent that avoids artifactual color generation in samples, which 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. Then FOX2 reagent was 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 aim was reached by incubating mitochondria at 37°C for 30 min in presence of 2 mM 2,2′-azobis(amidinopropane hydrochloride) (AAPH) a chemical free radical initiator.

Fatty Acid Composition

To obtain the fatty acid profile of mitochondrial membrane phospholipids, fatty acids methyl esters were formed according to the method of Lepage and Roy.[18] Briefly, 200 μg of mitochondrial protein were dissolved in 2 ml of methanol/benzene (4:1, v/v); 50 μg of the fatty acid 15:0 and 9 μM of BHT were added to the samples as internal standard and antioxidant, respectively. 200 μl of acetyl chloride were slowly added, then tubes were closed and subjected to methanolysis at 100°C for 1 h. After tubes were cooled in water, 5 ml of 0.43 M-K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged, the benzene upper phase was removed and transferred to other glass tube to be dried under nitrogen and resuspended to 100 μl with hexane. A gas-liquid chromatograph Model HP-5890 Series II (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector was used to analyze fatty acids. Chromatography was performed using a 60 m long capillary column; 32 mm id and 20 mm thickness impregnated with Sp™ 2330 FS (Supelco Incorporation, Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275°C respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Temperature programming (for a total time of 40 min) was as follows: initial temperature, 160°C for 5 min, 6°C/min to 195°C, 4°C/min to 220°C, 2°C/min to 230°C, hold 12 min, 14°C/min to 160°C. Individual fatty acids as well as total saturated (SFA), total unsaturated (UFA) total monounsaturated (MUFA), total polyunsaturated (PUFA) fatty acids and the unsaturation index (UI) were calculated on a percentage basis.

Protein and Phosphorus Determinations

The protein content of samples was measured by the method of Lowry *et al.*,[19] using bovine serum albumine as standard and phospholipid phosphorus was determined using the method of Marinetti.[20]

Statistical Analysis

The ANOVA test was used for each mitochondrial fraction and for each biochemical parameter tested. Homogeneity of variance was checked by Levene's test. When significant main differences were detected ($p < 0.05$), Scheffe's test was used for a *post hoc* comparison. All tests were performed using a personal computer Statistical Package (SPSS).

RESULTS AND DISCUSSION

The limited amount of samples available made it possible to investigate the CoQ_n contents of FM only. In the present study, we found that levels of CoQ_9 (1.09 ± 0.05 nmol/mg) CoQ_{10} (0.56 ± 10.03 nmol/mg) and the summation of CoQ_9 plus CoQ_{10} (1.66 ± 0.05 nmol/mg) were higher (between 10 and 20%, depending on the homologue) than those found in other studies regarding right-side cerebral cortex mitochondria.[2] CoQ_{10}/CoQ_9 ratio was 0.52, practically identical to that calculated in previous studies for rats of the same age; since the CoQ_{10}/CoQ_9 ratio remains constant regardless of the brain area and the mitochondrial fraction considered, it seems that such ratio could be critical for minimal efficiency of the mitochodrial machinery.[5]

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[6] and even more significance should be given to this fact when subcellular investigations are made.[2–4,6,10] Evidence was found supporting a biochemical diversity between perikaryal and intra-synaptic mitochondria[13] and specific attention was devoted to the microheterogeneity of intrasynaptic mitochondria.

The results of previous investigations (i.e. loss of antioxidant molecules, decrease in several enzymatic activities, etc.) suggest that HM may represent an aged mitochondrial population characterized by a partial impairment of typical mitochondrial functions.[2–4] The results of the present work may sustain the above-mentioned hypothesis. As it has been shown in Table I, HM held the highest HP contents (0.15 ± 0.01 μmol/mg), about 30% more than FM and LM ($p < 0.05$); HP in LM were also

TABLE I Basal and AAPH-induced levels of hydroperoxides and the ratio between induced and basal levels (I/B) in left side cerebral cortex synaptic and non-synaptic mitochondria from 4 months of age old rats. Values are mean ± EEM

	FM ($n = 8$) a	LM ($n = 5$) b	HM ($n = 6$) c	Statistical analysis ($P < 0.05$)
Basal (μmol/mg)	0.09 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	a vs. c; b vs. c
AAPH-induced (μmol/mg)	0.19 ± 0.02	0.14 ± 0.02	0.05 ± 0.01	a vs. c; b vs. c
Induced/Basal (I/B)	2.08 ± 0.09	1.25 ± 0.04	0.32 ± 0.06	a vs. b,c; b vs. c

TABLE II Mitochondrial fatty acid profile (expressed as % of total fatty acids). Data are means ± SEM

	FM ($n = 8$) a	LM ($n = 5$) b	HM ($n = 6$) c	Statistical analysis ($P < 0.05$)
16:0	34 ± 0.2	38.3 ± 0.6	46.4 ± 6.3	–
18:0	34.4 ± 0.3	29.4 ± 1.2	10.1 ± 2.1	c vs. a,b
18:1$n-9$	11.4 ± 0.1	10.4 ± 0.5	8.6 ± 1.4	–
18:2$n-6$	2.1 ± 0.1	2.0 ± 0.1	n.d.	–
18:3$n-3$	0.7 ± 0.1	1.0 ± 0.1	n.d.	–
20:4$n-6$	1.6 ± 0.1	1.8 ± 0.2	3.2 ± 0.5	c vs. a,b
20:5$n-3$	0.9 ± 0.1	1.7 ± 0.2	n.d.	a vs. b
22:4$n-6$	1.6 ± 0.1	1.7 ± 0.1	4.5 ± 0.8	c vs. a,b
24:1$n-9$	0.9 ± 0.1	1.0 ± 0.2	2.6 ± 0.6	c vs. a,b
22:5$n-6$	1.1 ± 0.1	1.0 ± 0.3	6.9 ± 1.6	c vs. a,b
22:6$n-3$	1.3 ± 0.1	1.2 ± 0.1	7.7 ± 1.9	c vs. a,b
SFA	71.5 ± 0.4	71.4 ± 0.9	64.2 ± 6.7	–
UFA	28.5 ± 0.4	28.6 ± 0.9	35.8 ± 6.7	–
MUFA	14.2 ± 0.2	14.0 ± 0.5	11.1 ± 1.9	–
MUFA:PUFA	1.2 ± 0.1	1.1 ± 0.1	0.4 ± 0.0	c vs. a,b
PUFA ($n-6$)	8.5 ± 0.2	9.1 ± 0.7	14.6 ± 2.7	c vs. a
PUFA ($n-3$)	4.6 ± 0.2	5.5 ± 0.5	9.8 ± 2.3	c vs. a
UI	0.9 ± 0.1	1.0 ± 0.1	88.5 ± 16.9	c vs. a,b

n.d.: not detectable; SFA: Saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UI: unsaturation index.

higher than in FM but the difference was small. The extent of AAPH-induced peroxidation differed among the various kinds of mitochondria with the highest values found in FM and the lowest ones in HM ($p < 0.05$ for FM vs. LM, FM vs. HM and LM vs. HM). In fact, FM suffered a HP increment of 0.19 μmol/mg protein. The effects produced by AAPH are even more evident in when the ratio between induced HP values, due to the AAPH action, and basal HP values (induced/basal (I/B) ratio) is taken into account. Thus, I/B for HM was 26% and 15% of the corresponding ratio LM and FM ($p < 0.05$) Such parameter can be an useful tool for giving an indication about the proneness of sample to be peroxidized: the higher the ratio, the higher its "peroxidative susceptibility". Also LM evidenced a lower susceptibility to oxidation than FM ($p < 0.05$). As HM fraction is less susceptible to peroxidation this might appear somewhat contradictory with its high content of basal HP. Such low *in vitro* vulnerability could depend on the fact that this mitochondrial population already presents a heavily compromised lipid phase; furthermore, as we will comment later, the presence of fatty acids is impaired, so there is less substrate to be peroxidized.

Very significant differences in the fatty acid profile were found among mitrochondrial subpopulations. These differences regarded both individual fatty acids and the most commonly used and useful indexes derived from fatty acid composition analysis, which were significantly modified in FM, or LM, compared to HM (Table II). Moreover, very considerable differences were found in fatty acid contents among the mitochondrial subpopulations. The total fatty acid amount in HM was only 20% of that assayed in the corresponding FM or LM subpopulations. In fact, if the fatty acid amount of FM fraction is taken as a reference value, the HM fractions

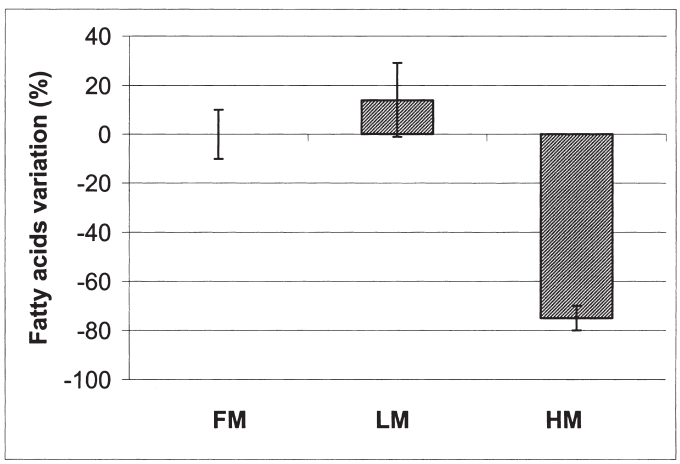

FIGURE 1 Total fatty acids variation in left side cerebral cortex synaptic and non-synaptic mitochondria from 4 months of age old rats. Data are expressed as percentage (%) variations versus non-synaptic (FM) fraction taken as reference. Values are means ± SEM.

displayed decreases of 75% or more (Fig. 1). On the other hand, the analysis of phospholipid phosphorus revealed that no differences in phosphorus content were present in any of the mitochondrial subpopulations assayed, therefore these data suggest a loss of fatty acids (or part of them because fatty acids shorter than C14:0 were not detectable by the method employed) by the phospholipid molecules. Moreover, the remaining fatty acids resulted more unsaturated, as it can be seen for the higher proportion of 20:4$n-6$, 22:4$n-6$ or 22:6$n-3$ found in HM in comparison with FM and LM. The MUFA/PUFA ratio, which is usually an index of the membrane susceptibility to peroxidation, was about one third in HM compared to LM or FM. A correlation between MUFA/PUFA index in membrane and the ability of the latter to withstand lipid peroxidation has been previously found in different conditions causing oxidative stress[21,22] and this index could even be more important than the net PUFA content itself. Several more indexes were also significantly modified, some of them in a dramatic manner, as in the case of UI, which was found to be three orders of magnitude greater in HM vs. LM.

The high proportion of PUFA in HM mitochondria could be part of a higher regulation mechanism. In this sense, it has been described how mitochondrial membranes are able to modify their fatty acid profile to maintain a constant membrane fluidity.[23–25] The mechanism proposed, which involved the partial substitution of saturated fatty acids in the membrane by PUFA, could be a consequence of the previously reported loss in enzymatic activities in HM mitochondria due to the aging imbalance in this population. Summarizing, the mitochondria could modify the composition of their membrane in order to maintain the functionality of the same. However, these changes are not enough to counterbalance the accumulated damage, indeed they contribute to the same by increasing the oxidisability of the membrane.

HM actually showed the highest content of native HP. In other words, they appeared to be the most oxidatively prejudiced among the subpopulations studies. The fact that HM are the most damaged mitochondria could fit perfectly with the very low enzymatic activities and low antioxidant contents typical of this subpopulation. The efficiency of antioxidant and repair mechanisms may be limited with age and the large extent of modifications occurring in HM at a membrane level cannot be completely repaired giving rise to an accumulation or injuries. A highly oxidized, functionally jeopardized, physically heavy organelle could be the final result of the progressive augmentation of noxious during the life-span of synaptic HM mitochondria.

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