Decrease of rotenone inhibition is a sensitive parameter of complex I damage in brain non-synaptic mitochondria of aged rats

Maria Luisa Genova[a], Carla Bovina[a], Mario Marchetti[a], Francesco Pallotti[b], Catleen Tietz[b], Graziella Biagini[c], Armanda Pugnaloni[c], Claudio Viticchi[d], Antonella Gorini[e], Roberto Federico Villa[e], Giorgio Lenaz[a,*]

[a]*Department of Biochemistry, University of Bologna, via Irnerio 48, 40126 Bologna, Italy*
[b]*Institute of Histology, University of Bologna, via Belmeloro 8, 40126 Bologna, Italy*
[c]*Institute of Human Morphology, University of Ancona, via Tronto 10/a, 60020 Torrette di Ancona, Italy*
[d]*INRCA, via S. Margherita 5, 60124 Ancona, Italy*
[e]*Institute of Pharmacology, University of Pavia, p.zza Botta 11, 27100 Pavia, Italy*

Received 8 April 1997; revised version received 5 May 1997

Abstract We investigated NADH oxidation in non-synaptic and synaptic mitochondria from brain cortex of 4- and 24-month-old rats. The NADH oxidase activity was significantly lower in non-synaptic mitochondria from aged rats; we also found a significant decrease of sensitivity of NADH oxidation to the specific Complex I inhibitor, rotenone. Since the rotenone-binding site encompasses Complex I subunits encoded by mtDNA, these results are in accordance with the mitochondrial theory of aging, whereby somatic mtDNA mutations are at the basis of cellular senescence. Accordingly, a 5 kb deletion was detected only in the cortex of the aged animals.

Key words: Aging; Brain mitochondria; Complex I; Rotenone; Mitochondrial DNA

1. Introduction

The 'mitochondrial theory of aging' [1] proposes that accumulation of somatic mutations of mitochondrial DNA (mtDNA), induced by continual exposure to free-radical attack leads to errors in the mtDNA-encoded polypeptide chains. The consequence of these alterations, which affect exclusively the four mitochondrial enzymic complexes involved in energy conservation, would be defective electron transfer and oxidative phosphorylation.

It is predicted that the highest frequency of mutations would affect Complex I (NADH–Coenzyme Q reductase) of the respiratory chain, for which seven mitochondrial genes are present [2]. Moreover, the deletions described to increase in aging in both humans and experimental animals, such as the 5 kb 'common' deletion, usually encompass a region containing genes for Complex I subunits [3].

Several studies reported decreases of NADH oxidative activity in mitochondria from different tissues [4]. However, the data available on the activity of Complex I in the nervous tissue in aging are scant and of difficult interpretation (cf., [5–7]).

The medical interest in Complex I recently increased because of its involvement in a number of genetic and acquired degenerative diseases. Of special interest is Leber's hereditary optical neuropathy (LHON), a maternally inherited disease associated to mtDNA point mutations; the most frequent form of LHON (with a mutation in nucleotide position 11 778 affecting the ND4 subunit of Complex I) is associated to increased resistance to rotenone [8], a classical inhibitor of Complex I that binds to the hydrophobic subunits of the enzyme. It was recently found in our laboratory that a similar increased resistance to rotenone is present in platelet membranes from old individuals [9].

One main problem involved in Complex I activity determination was recognized in our laboratory to be its systematic undervaluation because of the inadequacy of the quinone acceptors used for the assay [10]; this inadequacy led to the incapability to recognize even large differences in activity in tissues from aged rats [11]. It was also recognized that the assay of aerobic oxidation through the whole respiratory chain represents a better way to investigate NADH–CoQ reductase activity than the direct enzymatic determination [11].

Measuring total aerobic NADH oxidation has the additional advantage of eliminating uncertainties on the extent to what Complex I activity must be decreased before the respiratory activity would be compromised. According to the metabolic control theory [12], this extent is a function of the degree of flux control that Complex I exerts over the global flux of respiration.

In this study, we have investigated the properties of Complex I of synaptic and non-synaptic mitochondria obtained from the brain cortex of young and old rats.

2. Materials and methods

Chemicals, including decyl-ubiquinone (DB), were purchased from Sigma-Aldrich S.r.l., Milan, Italy. CoQ_1 was a kind gift from Eisai Co., Tokyo, Japan.

Two groups of Wistar male albino rats, aged 4 and 24 months, were kindly provided from an inbred colony by INRCA, Ancona, Italy.

For electron microscopy analysis, specimens of brain cortex from frontal sections were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% OsO_4 in the same buffer, dehydrated in a graded series of ethanol, transferred to propylene oxide and then embedded in Araldite (Fluka, Buchs, Switzerland). For each animal three tissue blocks were considered; 2 μm semifine sections were cut by a LKB microtome, stained with toluidine blue and observed by a Leitz Ortoplan light microscope.

Thin sections (80 nm) were cut by a LKB ultracut microtome, counterstained by uranyl acetate and lead citrate and observed with a Philips CM 10 electron microscope at 80 kV. Morphometrical investigations were performed by a LEICA Quantimet 500 image analyzer using an interactive software. Only cellular sections showing nucleus were considered.

*Corresponding author. Fax: (39) 51-351217.
E-mail: lenaz@biocfarm.unibo.it

PII S0014-5793(97)00638-8

Table 1
Morphometrical parameters in rat brain cortex

	4 months old	24 months old
Mean cellular area (μm^2)		
Neurons	136.4 ± 33.7	147.6 ± 18.1
Astrocytes	101.8 ± 22.6	115.9 ± 20.2
Cellular numerical density N_v[a]		
Neurons	2.33 ± 0.44	1.23 ± 0.51[b]
Astrocytes	2.91 ± 0.56	2.90 ÷ 0.66
Mitochondrial volume density V_v		
Neurons	0.046 ± 0.016	0.073 ± 0.038
Astrocytes	0.011 ± 0.005	0.012 ± 0.005
Mitochondrial area/field[c]		
Neurons	14.30 (81%)	13.25 (77%)
Astrocytes	3.26	4.03

Values are expressed as means ± SD.
[a]Calculated as number of cells/field (field = 7646.84 μm^2). [b]$P < 0.05$.
[c]This value was obtained by multiplying mitochondrial volume density × cellular numerical density. The number in parentheses is the relative mitochondrial area attributed to neurons with respect to the total mitochondrial area of combined neurons and astrocytes.

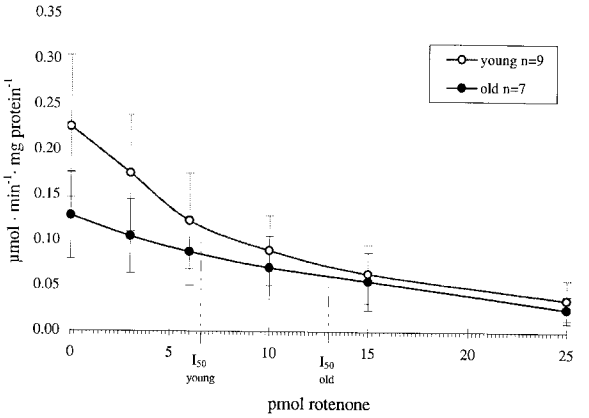

Fig. 1. Rotenone titration of the NADH oxidase activity in non-synaptic mitochondria from rat brain cortex. In the abscissa, the rotenone titer per mg mitochondrial protein was corrected for the content of active Complex I as expressed by NADH–ferricyanide activity [15].

Images from semifine sections at 40× magnification were used to evaluate astrocytes and neurons mean cellular area and cellular numerical density (N_v), calculated as number of cells/field (field = 7646.84 μm^2).

Twenty micrographs from each specimen (5 old and 5 young animals) were collected at a final magnification of 24 500× and processed for calculating the mitochondrial volume density (V_v) using the formula:

$$V_v = A_t / A_c$$

where A_t is the total mitochondrial area and A_c is the total cytoplasmic area. This stereological parameter expresses the volume fraction of mitochondria per unit volume of cytoplasm [13].

Free non-synaptic mitochondria (FM) and synaptic light (LM) and heavy (HM) mitochondria were purified from brain cortex of single animals as described in [14] except that protease inhibitors were omitted. Mitochondria were stored at −80°C. Before assays, samples were thawed and pulse sonicated 5 times at 10 s periods (150 W) with 50 s intervals, in an ice-water bath under nitrogen gas, to allow complete accessibility of substrates to the enzymes of the inner membrane.

NADH oxidation was measured following the decrease of absorbance of NADH at 340 minus 380 nm using an extinction coefficient of 5.5 mM^{-1} cm^{-1}. NADH–ferricyanide reductase activity was assayed measuring ferricyanide reduction at 420 minus 500 nm (ε=1 mM^{-1} cm^{-1}). The specific activity of ferricyanide reduction was used as a parameter proportional to the content of active Complex I in the membrane [15]. Enzymatic activities were assayed at 30°C, as described in [10].

In order to study mtDNA deletions, the total DNA was extracted from frozen specimens of frontal cerebral cortex. Samples were then treated with two different sets of primers so that two separate regions of the mitochondrial DNA were amplified by PCR. The first couple of primers, L7825 (nt 7825–7844) and H13117 (nt 12997–13117) [16], detects a deletion of 4.8 kb involving a 16 bp direct repeat that is similar in size and location to the 5.0 kb deletion observed in human mtDNA [17]. The second set of primers, L4395 (nt 4395–4418) and H5164 (nt 5145–5164), amplifies a region containing the light strand origin of the mtDNA [16]; for this reason it was employed in order to have a control of the wild-type sequence, confirming the presence of mtDNA in total DNA samples (data not shown).

Statistical investigations were performed by Student's *t*-test.

3. Results and discussion

Table 1 summarizes some histological features of the brain cortical tissue of young and old rats. Neither neurons nor glial cells showed deep morphological changes in terms of mean cellular area.

On the contrary, significant loss of neurons ($P < 0.05$) was observed in aged rats, while no changes of glial cells were found. Volumetric density of mitochondria (V_v) was not significantly different in neurons and astrocytes of the two groups.

Knowing the cellular numerical density (N_v), the mean cellular area and the relative content of mitochondrial surface (V_v) for either neurons and astrocytes, we can roughly calculate the total content of mitochondria, as mitochondrial area/field, and the relative proportion of those of glial and neuronal derivation. It can be shown that about 80% of mitochondrial content in the cell bodies is of neuronal origin both in young and old animals, in spite of the neuron decrease in the old.

This finding was used to estimate the relative content of neuronal and glial mitochondria in the non-synaptic population of isolated mitochondria; we can conclude that most

Table 2
Biochemical parameters in non-synaptic mitochondria (FM) and in synaptic light (LM) and heavy mitochondria (HM) from rat brain cortex

	FM (4 months old) [9]	FM (24 months old) [7]	LM (4 months old) [9]	LM (24 months old) [7]	HM (4 months old) [9]	HM (24 months old) [7]
Mitochondrial yield (mg prot./g tissue)	4.30 ± 0.91	3.21 ± 0.95	3.29 ± 0.33	3.55 ± 0.71	4.91 ± 2.22	6.02 ± 1.87
NADH oxidase activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$	0.226 ± 0.078	0.127 ± 0.048[a]	0.117 ± 0.042	0.112 ± 0.057	0.108 ± 0.016	0.089 ± 0.013[a]
NADH–ferricyanide reductase ($\mu mol \cdot min^{-1} \cdot mg^{-1}$	4.92 ± 0.92	3.87 ± 0.72[a]	4.19 ± 0.63	4.83 ± 2.48	3.95 ± 0.50	3.99 ± 0.46
I_{50} of rotenone (pmol rot./mg protein)	34 ± 14	52 ± 26	40 ± 14	33 ± 11	25 ± 8	26 ± 9
I_{50} of rotenone (corrected)[b]	6.7 ± 1.8	13.0 ± 4.8[a]	9.4 ± 2.8	7.9 ± 4.6	6.3 ± 2.4	6.6 ± 2.4

Values are expressed as means ± SD. The number of experiments is shown in parentheses. [a]$P < 0.05$. [b](pmol rot./mg protein)/NADH–ferricyanide reductase.

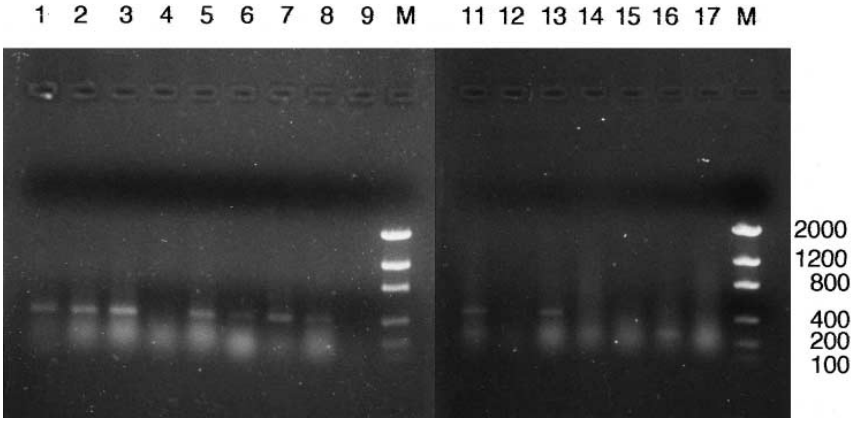

Fig. 2. PCR amplification of total DNA from rat brain cortex using primers designed for amplifying deleted mtDNAs. Primers employed: L7825 and H13117. Lanes 1–8 and lanes 11–13: 24-month-old rats. Lanes 14–17: typical samples from 4-month-old rats. Lane 9: negative control; M: molecular weight marker (Promega, Madison, WI). A band of 459 bp was found in 9 out of 11 24-month-old rats. This PCR product indicates the presence of a 4834 bp deletion. No amplification product was found in any of the 4-month-old rats (as exemplified in lanes 14–17).

mitochondria are of neuronal origin, even in the old animals, with important conclusions on the interpretation of the data.

The mitochondrial yield of the different fractions was estimated in terms of protein content; no changes were found in the two groups of animals (Table 2).

NADH oxidase activity showed a considerable decrease in the non-synaptic mitochondria population (FM) of aged rats (Table 2) with a corresponding loss of active Complex I content (as ascertained from the NADH–ferricyanide reductase activity (cf., [15]).

Sensitivity to rotenone, as shown in Fig. 1, was almost halved giving I_{50} values (the rotenone titer required to yield half inhibition) double in the 24-month-old rats with respect to controls ($P < 0.05$). The lack of a lag in the rotenone inhibition curves indicated that NADH–CoQ reductase is rate limiting for aerobic NADH oxidation.

No significant changes were found either in specific activity or in the rotenone sensitivity of the synaptic mitochondria except for a slight decrease of NADH oxidase activity in HM (Table 2).

The sensitivity to rotenone reflects the properties of the hydrophobic sector of Complex I and hence it may be diagnostic of alterations of the structure or assembly of those subunits or of their interaction with the membrane lipids. The contrast between the remarkable alterations of NADH oxidation properties in the free mitochondrial population and the lack of changes in the synaptic populations may find an explanation in the higher respiration rate observed in the FM fraction in this study as well as in previous investigations [18]. The mitochondrial theory of aging [1] states that tissues in which the respiration rate is high are more susceptible to the impairment of mitochondrial function. It is then possible that FM generate a higher proportion of oxygen reactive species inducing higher mitochondrial damage.

The genetic analysis of the mitochondrial DNA from frontal cerebral cortex revealed the presence of a 4834 bp deletion in 9 out of 11 aged rats (81.8%); no evidence of the mutation was found in any of the 10 young animals (0%) (Fig. 2).

This particular deletion is similar in size to the human 'common deletion' (4977 bp); it spans the same region of the mtDNA and it has been already detected in liver from old rats [17], showing an age-dependent increase.

Acknowledgements: This study was performed with grants from MURST, Rome.

References

[1] Miquel, J., Economos, A.C., Fleming, J. and Johnson, J.E. (1980) Exp. Gerontol. 15, 575–591.
[2] Tzagoloff, A. and Myers, A.M. (1986) Annu. Rev. Biochem. 55, 249–285.
[3] Schon, E.A., Sciacco, M., Pallotti, F., Chen, X., Bonilla, E. (1996) in: *Cellular Aging and Cell Death*, J. Wiley and Sons Inc., New York, 19–34.
[4] Ozawa, T. (1996) *Phys. Rev.* in press.
[5] Bowling, A.C., Mutisya, E.M., Walker, L.C., Price, D.L., Cork, L.C. and Beal, M.F. (1993) J. Neurochem. 60, 1964–1967.
[6] Fernandez, M.L., Martinez, M., De Juan, E., Diez, A., Bustos, G. and Miquel, J. (1994) Brain Res. 644, 355–383.
[7] Villa, R.F. and Gorini, A. (1991) Neuro. Chem. (Life Sci. Adv.) 10, 49–59.
[8] Degli Esposti, M., Carelli, V. and Ghelli, A. (1994) FEBS Lett. 352, 375–379.
[9] Merlo Pich, M., Bovina, C., Formiggini, G., Cometti, G.G., Ghelli, A., Parenti Castelli, G., Genova, M.L., Marchetti, M., Semeraro, S. and Lenaz, G. (1996) FEBS Lett. 380, 176–178.
[10] Lenaz, G., Fato, R., Genova, M.L., Formiggini, G., Parenti Castelli, G. and Bovina, C. (1995) FEBS Lett. 366, 119–121.
[11] Genova, M.L., Castelluccio, C., Fato, R., Parenti Castelli, G., Merlo Pich, M., Formiggini, G., Bovina, C., Marchetti, M. and Lenaz, G. (1995) Biochem. J. 311, 105–109.
[12] Davey, G.P. and Clark, J.B. (1996) J. Neurochem. 66, (4) 1617–1624.
[13] Weibell, E.R. (1979) in: *Stereological Methods: Practical Methods for Biological Morphometry* (Weibell E.L., Ed.), vol. I, pp. 9–62, Academic Press, New York.
[14] Battino, M., Gorini, A., Villa, R.F., Genova, M.L., Bovina, C., Sassi, S., Littarru, G.P. and Lenaz, G. (1995) Mech. Ageing Dev. 78, 173–187.
[15] Fato, R., Estornell, E., Di Bernardo, S., Pallotti, F., Parenti Castelli, G. and Lenaz, G. (1996) Biochemistry 35, 2705–2716.
[16] Gadaleta, M.N., Pepe, G., De Condia, G., Quagliariello, C., Sbisà, E. and Saccone, C. (1989) J. Mol. Evol. 28, 497–516.
[17] Edris, W., Burgett, B., Colin Stine, O. and Filburn, C.R. (1994) Mutation Res. 316, 69–78.
[18] Battino, M., Bertoli, E., Formiggini, G., Sassi, S., Gorini, A., Villa, R.F. and Lenaz, G. (1991) J. Bioenerg. Biomembr. 23, 345–363.