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Idebenone induces oxygen consumption rate modifications in aged rat brain mitochondria

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Abstract

Idebenone effects on oxygen consumption in brain mitochondria, obtained from young and aged rats, were evaluated. Sixty rats (3 and 20 months of age) were treated for 3 months with 30 mg/kg of idebenone and compared to a placebo group. Brain mitochondria oxygen consumption rate was measured by polarographic techniques in basal (State 4), ADP-stimulated (State 3) and uncoupled conditions. When Complex I substrates (pyruvate + malate) were used, aged non-treated rats showed a significant increase in State 4 (175%) and uncoupled (152%) O_2-uptake rate; no difference was found in State 3 respiration and in ADP/O_2 ratio. Idebenone was able to reverse these age-related effects probably acting on lipid peroxidation and the mitochondrial respiratory chain. No differences were found in mitochondrial enzymatic activities. Copyright © 1997 Elsevier Science Ireland Ltd.

Keywords: Idebenone; Brain mitochondria; Lipid peroxidation; Ageing

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1. Introduction

Recent studies suggest a possible role of lipid peroxidation and mitochondrial damage in physiological aging (Adelman et al., 1988; Bandy and Davison, 1990) as well as in the pathogenesis of cerebral degenerative diseases (Parker et al., 1990a,b; Schapira et al., 1990; Shoffner et al., 1991; Rosen et al., 1993). Thus, it is of great interest to study idebenone (6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone), a synthetic ubiquinone homologue, that is considered to function as an electron carrier in the respiratory chain and as an inhibitor of lipid peroxidation.

Idebenone is able to restore NADH and succinate oxidation activities in coenzyme Q-depleted canine brain mitochondria (Sugiyama et al., 1985).

Oxidized-idebenone, added in vitro to rat brain mitochondria, was found to decrease the State 3 respiration when glutamate was used as substrate; on the other hand, when reduced-idebenone was the substrate, a rapid oxygen consumption occurred, suggesting that reduced-idebenone is oxidized through an electron transfer to the ubiquinol-cytochrome *c* reductase (Imada et al., 1989).

Idebenone markedly inhibits NADH- and NADPH-dependent lipid peroxidation in brain mitochondria (Suno and Nagaoka, 1989), mainly if it is converted to the reduced form which was found to capture the $OH^{\cdot}$ free radicals with production of superoxide anion radicals (Zs-Nagy and Floyd, 1990).

Oxidized-idebenone, stimulating superoxide dismutase (SOD) synthesis, may be useful against the toxic action of free radicals (Nagy and Zs-Nagy, 1990). Furthermore idebenone was found to ameliorate cerebral glucose metabolism, suppressing the increase in lactate and the decrease in ATP in ischemic brain tissue (Nagaoka et al., 1989).

Idebenone improves the short-term memory impairment induced by a decreased cholinergic activity in animal models of vascular and Alzheimer types of dementia, as well as learning disabilities produced by serotonergic dysfunctions in rats (Yamazaki et al., 1989). Idebenone is commercialized in Japan for the therapy of cerebrovascular disorders (Donà et al., 1988); besides, it was found useful in the therapy of mitochondrial encephalomyopathies (Ihara et al., 1989; Yamazaki et al., 1991; Mashima et al., 1992).

The aim of this study is the evaluation of the efficacy of idebenone in improving mitochondrial oxygen utilization in aged rat brain measuring mitochondrial enzymatic activities and respiratory chain functions.

2. Materials and methods

2.1. Materials

Idebenone, a highly hydrophobic quinone type molecule, was synthesized by Cyanamid Italia S.p.A. It is sufficiently absorbed, crosses easily the blood brain barrier and experiences a fast metabolization. Its toxicity is very low and it is well tolerated in subacute and chronic treatments (Zs-Nagy, 1990).

All the other chemicals except ADP (Boeringher Mannheim) were from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals

Male Sprague–Dawley rats Ico: OFA-SD (I.O.P.S. Caw) from IFFA CREDO Colony (L'Arbresle, France) were used for all studies. We used 30 young rats (3-month-old, weight 500–550 g) and 32 aged rats (20-month-old, weight 550–600 g).

At the beginning of the study each group was divided into two subgroups: the former was treated with 30 mg/kg per os of idebenone, suspended in 5% arabic gum solution, and the latter was treated with equivalent amounts of the arabic gum solution without idebenone.

The animals were housed in single cages in an air-conditioned room (22°C ± 2°C). Certified standard diet, deionized water and good laboratory practice requirements were offered ad libitum.

Each animal was treated for 3 months. At the end of this period it was sacrified by decapitation and the brain was immediatly removed.

2.3. Mitochondria purification

Analyses were performed on fresh intact mitochondria (Clark and Nicklas, 1970) obtained from three pooled brains after the elimination of the cerebellum and forebrain. Subsequent manipulations were performed at 4°C.

The brains were minced and homogenized by hand in all-glass Dounce homogenizers (Kontes, USA) with an ice-cold homogenization buffer (250 mM Sucrose, 10 mM Tris, 0.5 mM EDTA-K_2, pH 7.4). The homogenate was centrifuged 3 min in a Beckman J2-21 refrigerated centrifuge at 2000 × *g* and then the supernatant 8 min at 12 500 × *g*. The pellet was resuspended in 3% Ficoll, layered on 6% Ficoll buffer (Ficoll 6%, mannitol 0.24 mM, sucrose 0.06 mM, EDTA-K_2 50 mM, Tris 10 mM, pH 7.4) and centrifuged 30 min at 11 500 × *g*; the pellet of isolated mitochondria was washed, centrifuged 10 min at 12 500 × *g* and then resuspended.

One sample of the isolated mitochondria was immediately used for polarographic measurements; other samples were frozen at − 20°C to perform enzymatic analyses.

2.4. Quality of mitochondrial preparations

The mitochondrial preparations were tested to guarantee similar characteristics in the four groups. Yield (mg of mitochondrial proteins/g of brain) and recovery (percentage of mitochondrial citrate synthase activity compared to total citrate synthase activity of brain homogenate) were measured.

Citrate synthase activity was measured before and after the addition of Triton X-100 (10%) to test the integrity of the inner mitochondrial membrane.

2.5. Mitochondrial enzymatic activities

Previously described spectrophotometric assays (Bresolin et al., 1985) were used to measure succinate cytochrome *c* reductase, succinate dehydrogenase, NADH dehydrogenase, NADH cytochrome *c* reductase (rotenone-sensitive), cytochrome *c* oxidase and citrate synthase activity on previously frozen samples of isolated rat brain mitochondria. A Perkin-Elmer lambda 5 spectrophotometer at 30°C was used.

2.6. Respiratory studies

Respiratory chain activities were measured polarographically at 28°C with a Gilson 5/6 Oxygraph using a Clark-type electrode (Clark and Nicklas, 1970). The respiration medium contained 150 mM mannitol, 50 mM sucrose, 200 mM KCl, 10 mM orthophosphoric acid, 40 mM Tris, 0.1 mM K_2-EDTA (pH 7.4), 0.5 mg bovine serum albumin and approximately 1 mg of mitochondrial protein. NAD-dependent substrates (pyruvate + malate, α-ketoglutarate + malate), FAD-dependent substrates (succinate with rotenone) and antimycin A (AA), provided with the artificial electron donors tetramethylphenylenediamine (TMPD) + ascorbate, allowed the evaluation of Complex I, II and IV, respectively.

Mitochondrial respiration was stimulated by adding 25 mM ADP. Oxygen uptake rate was measured before (State 4) and after (State 3) the addition of ADP; respiratory control index (RCI) was calculated as State3/State4 and ADP/O_2 ratio was calculated as the ratio between the added amount of ADP and the total oxygen consumption during State 3 respiration.

Uncoupled O_2 consumption was evaluated adding CCCP (carbamyl cyanide *m*-chlorophenylhydrazone) when pyruvate + malate were used as substrates.

Protein content was measured by the Lowry's method (Lowry et al., 1951).

Statistic analyses were carried out by the Student's *t*-test.

3. Results

3.1. Effect of treatment

Idebenone treatment was well tolerated. Two aged rats were withdrawn from the study because they developed tumours.

3.2. Quality of mitochondrial preparations

We prepared fresh intact rat brain mitochondria with a good yield (2.15–2.75 mg/g brain) and recovery (10–17% of the initial brain citrate synthase activity).

Samples had low citrate synthase activity before adding Triton X-100, with a significant (11–19-fold) increase after Triton X-100 addition (1364 ± 166 nM/min/mg) (Table 1) as it was expected in a good mitochondrial preparation.

Table 1
Comparison of mitochondrial preparations from rat brain

		Mitochondria					Homogenate		
		Yield	Recovery (%)	Protein content	Citrate synthase	Triton increase	Protein content	Citrate synthase	Triton increase
Young placebo	Mean	2.54	13.90	11.15	1403	15.55	10.78	287	26.95
	S.D.	0.41	4.07	2.21	129	3.37	1.55	18	7.64
Young treated	Mean	2.22	13.08	10.10	1418	15.82	11.28	278	26.53
	S.D.	0.19	2.07	0.98	52	3.27	1.07	19	5.59
Aged placebo	Mean	2.55	16.40	12.00	1355	16.12	10.10	257	22.73
	S.D.	0.21	2.71	1.18	55	4.36	1.40	11	1.42
Aged treated	Mean	2.43	13.35	11.53	1444	17.33	10.58	271	29.78
	S.D.	0.25	4.28	1.23	154	4.48	2.34	27	9.81

Yield = mg of mitochondrial/g of fresh brain.

$$\text{Recovery} = \frac{\text{mitochondria citrate synthase activity}}{\text{homogenate citrate synthase activity}} \times \text{Yield}$$

Citrate synthase activity was measured after Triton X-100 addition and expressed as nmol/min/mg of proteins. The effect of Triton X-100 addition is expressed as fold of increase in enzymatic activity. Protein content is expressed as mg of non-collagen proteins per ml.

3.3. Oxygen consumption rate (Table 2; Figs. 1 and 2)

3.3.1. Placebo vs. treated young rats

No difference was found.

3.3.2. Young vs. aged placebo rats

Using pyruvate + malate as substrates there was an increase in State 4 respiration (175%) in aged placebo rats (19.02 ± 1.90) compared to young rats (10.87 ± 3.28) (Fig. 2); this difference was significant ($P < 0.001$). Only a slight increase was found in State 3 respiration (116%), thus the RCI was decreased (67%) in aged (7.52 ± 1.51) compared to young rats (11.25 ± 2.43) with a $P < 0.02$.

Table 2
Polarographic analyses on rat brain mitochondria

	State 4	State 3	RCI	ADP/O_2	CCCP
Pyruvate + malate					
Young rats					
Placebo	10.87 ± 13.28	121 ± 15.9	11.25 ± 2.43	4.24 ± 0.3	147 ± 24
Idebenone	14.03 ± 5.43	128.7 ± 6.6	10.2 ± 4.14	4.02 ± 0.1	174 ± 25
Aged rats					
Placebo	19.02 ± 1.90**	141 ± 19.1	7.52 ± 1.51***	4.21 ± 0.38	224 ± 38*
Idebenone	13.03 ± 3.42*	138.3 ± 20.4	11.85 ± 2.77***	4.29 ± 0.4	187 ± 50
α-Ketoglutarate + malate					
Young rats					
Placebo	14.75 ± 6.45	103.8 ± 18.42	7 ± 2.23	3.8 ± 0.35	
Idebenone	11.72 ± 2.51	109 ± 15	8.7 ± 2.18	3.82 ± 0.33	
Aged rats					
Placebo	17.7 ± 5.14	122.6 ± 13.22	7.38 ± 2.03	3.58 ± 0.35	
Idebenone	13.02 ± 5.97	118.67 ± 17.99	10.58 ± 4.32	3.73 ± 0.32	
Rotenone + succinate					
Young rats					
Placebo	40.35 ± 5.37	136.4 ± 19.92	3.4210.48	2.44 ± 0.3	
Idebenone	42.72 ± 7.52	149.6 ± 20.37	3.610.67	2.58 ± 0.14	
Aged rats					
Placebo	45.68 ± 2.94	159.6 ± 13.06	3.5210.27	2.35 ± 0.1	
Idebenone	38.34 ± 7.08	152.2 ± 25.53	3.9210.53	2.49 ± 0.12	
Anti-A + TMPD + ascorbate					
Young rats					
Placebo		51.3 ± 5.43			
Idebenone		55.74 ± 8.8			
Aged rats					
Placebo		50.98 ± 2.03			
Idebenone		53.74 ± 9.77			

**$P < 0.001$ State 4 placebo young-aged.
*$P < 0.01$ State 4 aged placebo-treated/CCCP placebo young-treated.
***$P < 0.02$ RCI placebo young-treated/RCI aged placebo-treated.
Values are expressed as nAtoms O_2/min/mg of mitochondrial proteins.

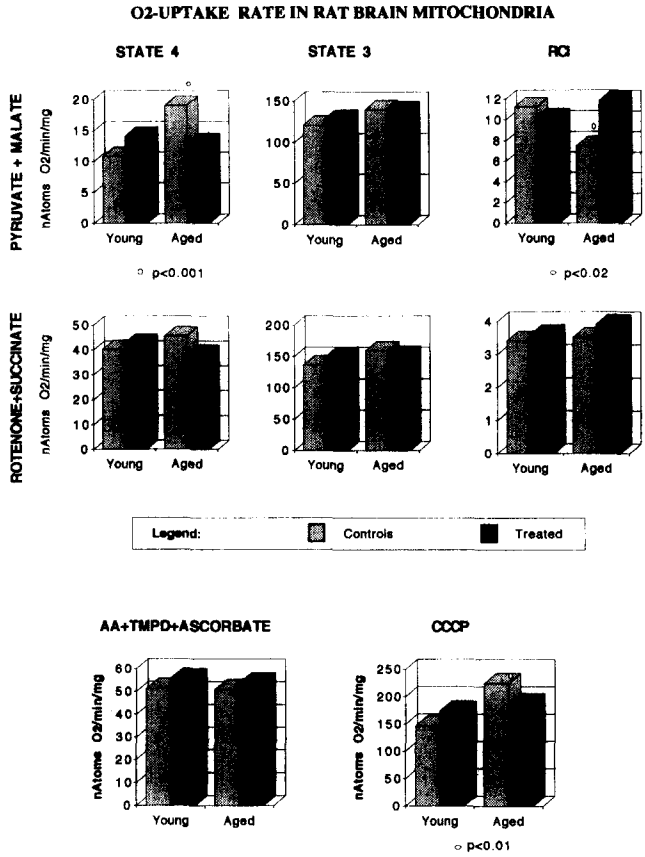

Fig. 1. Effect of idebenone treatment on Complex I, Complex II, Complex IV activities, and uncoupled (CCCP) respiration.

Uncoupled O_2-uptake rate was higher (152%) in aged rats (224 ± 38) than in the young ones (147 ± 24) and the difference was significant ($P < 0.01$).

No age-related effect was found in ADP/O_2 ratio (99%). State 4, State 3, RCI and ADP/O_2 presented no age-related difference when α-ketoglutarate + malate, succinate or TMPD + ascorbate were used.

3.3.3. Young placebo vs. aged treated rats

Idebenone treatment was effective in normalizing State 4 (119% of young rats), CCCP respiration (127%) and RCI (105%) when pyruvate + malate were used as substrates. It is evident that idebenone-treated rats showed a better respiratory control and a decreased extramitochondrial oxygen consumption.

3.3.4. Placebo vs. treated aged rats

A relevant difference was noted between treated and control groups of aged rats when State 4 respiration ($p < 0.01$) or RCI ratio ($p < 0.02$) were studied.

3.4. Mitochondrial enzyme activities

Mitochondrial enzyme activities were very similar for each group and no statistic differences were found (Table 3).

4. Discussion

In this study we were able to demonstrate, for the first time, the effects of aging on cerebral respiration in rats, measuring the oxygen consumption on fresh intact cerebral mitochondria.

Aging is associated with an increased level of mitochondrial DNA alterations (Cortopassi and Arnheim, 1990; Corral-Debrinski et al., 1992; Tritschler and Medori, 1993). Pikò et al. (1988) found 5% of mutated mitochondrial DNA in aged rats, focusing attention on the possible role of genetic mitochondrial alterations in aging. As a matter of fact the oxygen consumption rate of human muscle mitochondria shows a negative correlation with age (Cooper et al., 1992).

Several pathogenic noxae can damage mitochondria directly through the impairment of the oxidative-phosphorylation system (drugs, toxic), or injure mitochondrial DNA (drugs, radiations, free radicals) or mitochondrial membranes (free radicals).

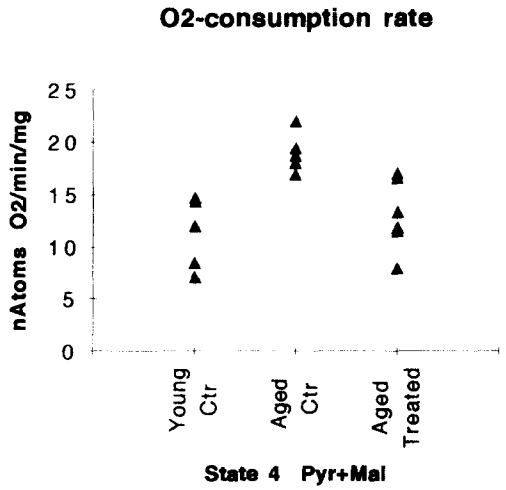

Fig. 2. Oxygen consumption rate with pyruvate + malate. The results of five measurements for each group are compared.

Table 3
Rat brain mitochondrial enzymatic activities

		NADH dehydrogenase	NADH cytochrome *c* reductase	Rotenone inhibition (%)	Succinate dehydrogenase	Succinate cytochrome *c* reductase	Cytochrome *c* oxidase	Citrate synthase	Cytochrome *c* oxidase/citrate synthase activity	Protein concentration (mg/ml)
Young rats (placebo)	Mean (5)	798.00	204.40	37.20	54.00	196.00	1530	1786	0.86	9.54
	S.D.	97.28	61.22	25.17	5.52	29.39	450	215	2.09	2.61
Young rats (idebenone)	Mean (5)	719.80	185.60	41.60	58.40	197.20	1372	1885	0.74	10.16
	S.D.	71.19	32.75	27.26	0.89	16.51	294	265	0.20	1.72
Aged rats (placebo)	Mean (5)	735.40	185.00	40.80	55.40	179.40	1468	1710	0.86	11.98
	S.D.	91.34	35.92	28.91	7.23	14.42	211	208	0.12	0.96
Aged rats (idebenone)	Mean (5)	724.33	192.33	41.00	57.00	179.67	1486	1749	0.84	11.55
	S.D.	120.73	33.51	32.17	7.10	18.38	240	193	0.17	1.26

NADH dehydrogenase; NADH cytochrome *c* reductase (rotenone action is expressed as % of inhibition effect); succinate dehydrogenase; succinate cytochrome *c* reductase; cytochrome *c* oxidase and citrate synthase enzymatic activity.
Values are expressed as nmol/min/mg of proteins.

Great attention was paid to the toxic action of free radicals and lipid peroxidation which are thought to be mainly derived from the mitochondrial respiratory chain. A reduced respiratory function may increase free radical levels and severely damage the mitochondria and the cell; this phenomenon is particularly evident in the brain because of the high lipid content of the membranes. Free radical production has been demonstrated to increase with age (Adelman et al., 1988; Bandy and Davison, 1990) whereas reduced glutatione (GSH) levels decrease. A correlation between the level of SOD and age has also been reported (De Lustig et al., 1993). These data represent an important basis to explain the described effects of idebenone on brain mitochondria of aged rats. Our study shows that idebenone may damp the described age-related alterations. In fact, the increase of State 4 respiration and the decrease of RCI in aged rats show that aged mitochondria have a continuous oxygen consumption, which is not always associated with ADP phosphorylation, since the increase in state 4 reflects the expression of a greater mitochondrial oxidative/uncoupled activity, which is unfavorable from the bioenergetic point of view. Idebenone normalizes this phenomenon restoring a normal balance. As a matter of fact, the ADP/O_2 ratio is unchanged according to previously by reported data (Sugiyama and Fujita, 1985); moreover uncoupled respiration (CCCP) was higher in aged rat mitochondria. It seems likely that aging results in a greater oxygen consumption that is unrelated to phosphorylation and to energy production as occurs during lipid peroxidation (Sugiyama and Fujita, 1985).

These results are in agreement with the previously reported idebenone induced improvement of learning and memory in rats (Yamazaki et al., 1989); in fact these functions usually deteriorate with age. It seems that the biochemical modifications produced by the drug have definite functional and behavioural consequences.

Ageing causes no effect on oxygen uptake when substrates other than pyruvate + malate were used. Giving the fact that Complex I has several subunits codified by mitochondrial DNA, which is more vulnerable to injuries than nuclear DNA, it is a favourite site of action of several toxics. Glutamate + malate enter the respiratory chain through Complex I as well as pyruvate + malate do, but this system is usually less sensitive to inhibition.

The mitochondrial electron carrier properties and the antiperoxidant functions of idebenone, which are closely related, may be responsible for the modifications in oxygen consumption induced by the drug. Probably the action on lipid peroxidation is the most relevant one, since State 4 and CCCP respiration are the most affected parameters in our study.

In an acute experiment, dealing with the same parameters, rats treated with 100–300 mg of idebenone for 3 days, showed a little increase in State 3 respiration in addition to the effect on the State 4 (Sugiyama and Fujita, 1985). As it would be expected, this increase, that was not confirmed by our study, could be directly related to the idebenone effect on the mitochondrial respiratory chain.

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