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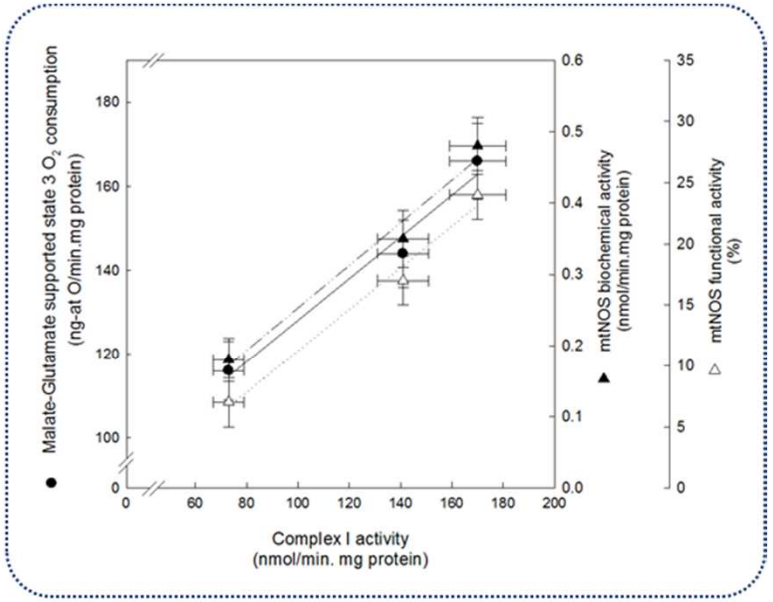
Parkinson´s disease model

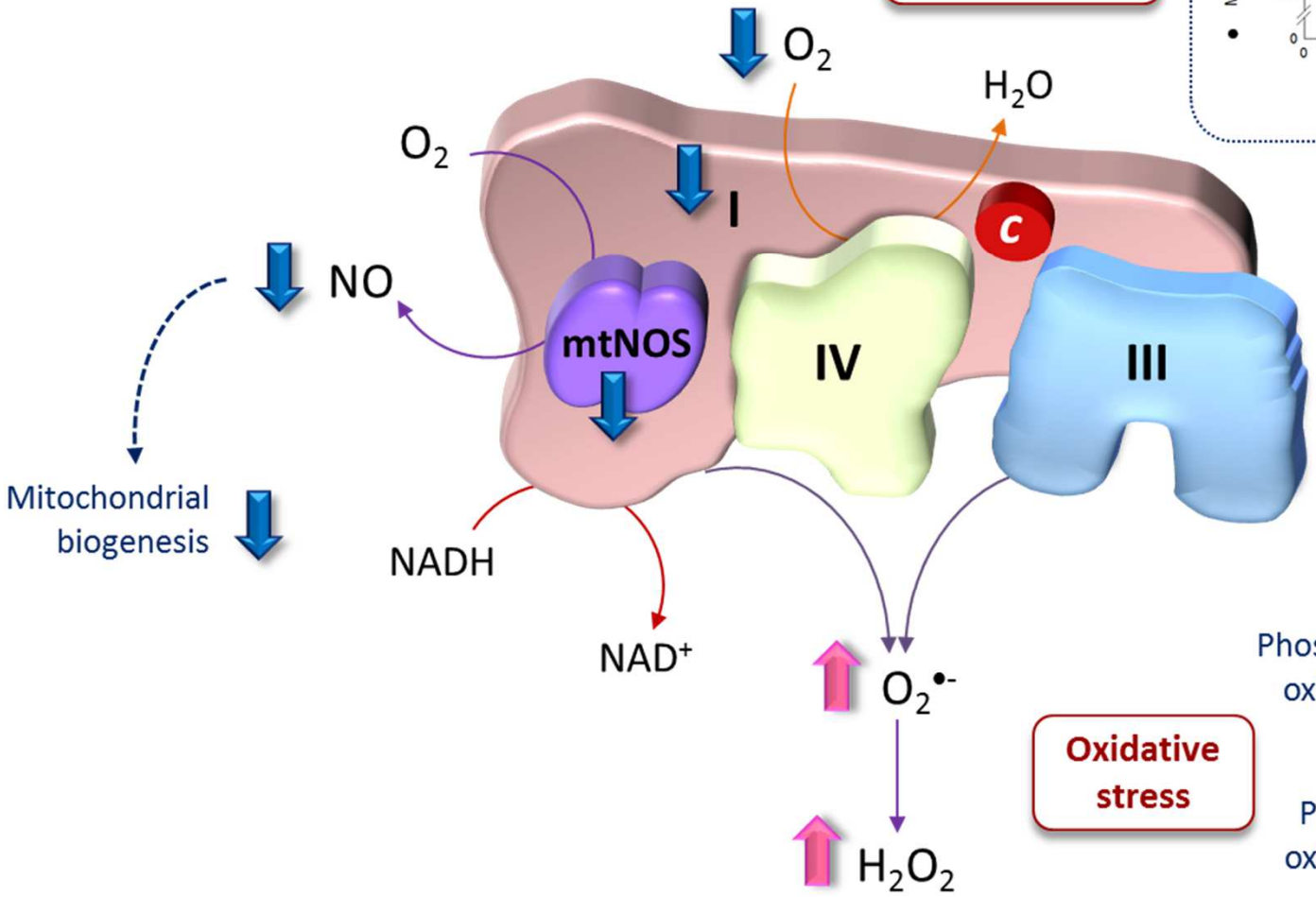
Rotenone, i.p., 30 or 60 days

Striatum
Frontal cortex

Complex I syndrome

Dysfunctional mitochondria





Oxidative stress

Phospholipid oxidation

Protein oxidation

Research Article

COMPLEX I SYNDROME IN STRIATUM AND FRONTAL CORTEX IN A RAT MODEL OF PARKINSON DISEASE

Laura B. Valdez[1,2,*], Tamara Zaobornyj[1,2], Manuel J. Bandez[3], José María López-Cepero[4], Alberto Boveris[1,2] and Ana Navarro†[3]

[1]University of Buenos Aires, School of Pharmacy and Biochemistry, Physical Chemistry Division, Buenos Aires, Argentina.

[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Institute of Biochemistry and Molecular Medicine (IBIMOL, UBA-CONICET), Buenos Aires, Argentina.

[3]University of Cadiz, School of Medicine, Department of Biochemistry and Molecular Biology, Cadiz, Spain.

[4]University of Cadiz, School of Medicine, Department of Cell Biology and Histology, Cadiz, Spain.

† Passed away on January 25, 2011. This *In memoriam paper* is homage to Ana Navarro and her contributions to free radical biology, in the 8th anniversary of her passing.

Running head: Brain mitochondrial dysfunction in rotenone-treated rats

***Corresponding author:** Laura B. Valdez, Cátedra de Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina. Tel: 54-11-5287-4235. E-mail: lbvaldez@ffyb.uba.ar

ASTRACT

Mitochondrial dysfunction named complex I syndrome was observed in striatum mitochondria of rotenone treated rats (2 mg rotenone/kg, i.p., for 30 or 60 days) in an animal model of Parkinson disease. After 60 days of rotenone treatment, the animals showed: (a) 6-fold increased bradykinesia and 60% decreased locomotor activity; (b) 35-34% decreases in striatum O_2 uptake and in state 3 mitochondrial respiration with malate-glutamate as substrate; (c) 43-57% diminished striatum complex I activity with 60-71% decreased striatum mitochondrial NOS activity, determined both as biochemical activity and as functional activity (by the NO inhibition of active respiration); (d) 34-40% increased rates of mitochondrial $O_2^{\bullet-}$ and H_2O_2 productions and 36-46% increased contents of the products of phospholipid peroxidation and of protein oxidation; and (e) 24% decreased striatum mitochondrial content, likely associated to decreased NO-dependent mitochondrial biogenesis. Intermediate values were observed after 30 days of rotenone treatment. Frontal cortex tissue and mitochondria showed similar but less marked changes. Rotenone-treated rats showed mitochondrial complex I syndrome associated with cellular oxidative stress in the dopaminergic brain areas of striatum and frontal cortex, a fact that describes the high sensitivity of mitochondrial complex I to inactivation by oxidative reactions.

KEYWORDS

Brain mitochondria; complex I; rotenone; H_2O_2 production; NO production; mitochondrial NOS activity

ABBREVIATIONS

HbO_2: oxyhemoglobin

L-NAME: N^G-nitro-L-arginine methyl ester

L-NMMA: N^G-methyl-L-arginine

NOS: nitric oxide synthase

nNOS: neuronal nitric oxide synthase

mtNOS: mitochondrial nitric oxide synthase

PD: Parkinson´s disease

INTRODUCTION

Human brain requires a continuous energy supply that is expressed by brain O_2 consumption which accounts for 20% of the total O_2 taken up in spite that brain weight is only 2% of total body mass [1]. Mitochondrial complex I (NADH-ubiquinone reductase) is a large protein complex that transfers electrons from NADH to ubiquinone with the simultaneous pumping of H^+ from the mitochondrial matrix to the intermembrane space [2-4], an essential activity in the chemiosmotic mechanism of oxidative phosphorylation. The mitochondrial dysfunction with altered complex I activity has been named "complex I syndrome" [5], characterized by (a) decreases in tissue and mitochondrial O_2 consumptions, this latter observed only with NAD-dependent substrates; (b) reduction in complex I and in nitric oxide synthase (mtNOS) activities; and (c) increases in the rates of mitochondrial superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) production and in the contents of lipid peroxidation and protein oxidation products. It is understood that complex I syndrome describes the high sensitivity of mitochondrial complex I to inactivation by oxidative reactions. The mentioned mtNOS is the mitochondrial form of NOS, originally reported in rat liver mitochondria by Giulivi et al. [6-8] and later identified as the shorter α-variant of nNOS by Elfering et al. [9]. Mitochondrial NOS seems to be contiguously located to complex I being both enzymes functionally linked [10-13]. Particles from heart mitochondria produce NO by succinate-dependent reverse electron flow [13]. The association between mtNOS and complex I is compatible with the concept of respiratory chain supercomplexes with strong protein-protein interactions [14], and with the dependence of mtNOS activity on the metabolic states and on membrane potential [15,16].

Complex I dysfunction has been observed in Parkinson's disease [17,18], ischemia-reperfusion [12,19], endotoxic shock [20] and aging [21-23], accompanied by changes in mtNOS activity [12, 18-21]. In rat brain, mitochondrial NO production has been reported in cortex, hippocampus and striatum [24-26]. Nitric oxide is deeply involved in brain physiology and biochemistry by the NO-cGMP-dependent vasodilation [27], and by the inhibitions of cytochrome oxidase activity and of O_2 uptake [28, 29]. Other reported actions

for NO within the brain include promotion of mitochondrial biogenesis and regulation of calcium channels function [30,31].

Human Parkinson disease (PD) is a neurodegenerative disorder with clinical signs of neuromotor impairment: bradykinesia, rest tremor, muscular rigidity and a flexed posture [32,33]. The occurrence of motor and non-motor symptoms in PD raised the interest on the mitochondrial function in the involved dopaminergic brain areas. Striatum bodies, in short striatum, are bilateral areas of coordination and integration of involuntary movements located in the main pathway of motor impulses. Striatum has efferent connections to *substantia nigra* and to other midbrain nuclei [33] involved in processing emotions and in performing cognitive and psychological associations [34].

Animal models of PD have contributed to the knowledge of the biochemical and cellular characteristics of the disease. These models use toxins targeted to dopaminergic neurons and reproduce the motor symptoms of human PD [35-39]. Between those toxin, rotenone, is hydrophobic, accumulates in mitochondria and inhibits mitochondrial respiration. Therefore, this report deals with the mitochondrial function in striatum and in frontal cortex of rats chronically treated with rotenone. The two mentioned brain areas show “complex I syndrome” and oxidative stress, two conditions that seem to be involved in the motor and non-motor manifestations of human PD.

MATERIALS AND METHODS

1. Drugs and chemicals

Rotenone and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade.

2. Experimental design

2.1. Animals

Male Wistar rats (270 ± 12 g, 3 months old) from the Department of Experimental Animals of the University of Cadiz were housed at 22 ± 1 °C, with 12 h light/dark cycles, and with full access to water and food. Experiments were carried out in accordance with the Guiding Principles for Research Involving Animals of the American Physiological Society, the Guidelines of the European Union Council (86/609/CEE), and the Spanish regulations for the use of laboratory animals (BOE 67/8509-12, 1988).

2.2. Rotenone treatment

Rotenone injections (2.0 mg/kg rat, i.p., 6 days a week) were given for 30 or 60 days as described by Alam and Schmidt [37,38]. Rats were randomized into 3 groups of 15 rats each: control, 30 days-rotenone, and 60 days-rotenone. Animals were weighed prior to injection. Control rats were injected with vehicle (olive oil).

3. Neurologic and locomotive tests

The tests of catalepsy and of open field activity were performed weekly. In the catalepsy test, aimed to evaluate bradykinesia and rigidity, each rat was hung by the paws on a vertical grid (25 cm wide and 45 cm high with 1 cm between wires). The time taken to move one of the paws was determined. The open-field test was used to assess the spontaneous locomotive activity. Animals were placed in a 50 × 50 cm box with 45 cm

walls. The frequency which rats stood up on their hind legs and the total standing time during 10 min were recorded [37].

4. Isolation of striatum and frontal cortex mitochondria

Rats were killed by decapitation, brain was quickly removed after opening the skull and striatum and frontal cortex were immediately excised [24,40]. Striatum and frontal cortex from 5 rats were pooled for mitochondrial isolation, that was performed as described [24,41]. Tissue fragments were washed and homogenized in 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA and 10 mM Tris-HCl, pH 7.40, at 9 ml of homogenization medium/g of brain in a small Potter homogenizer with a Teflon pestle. Homogenates were centrifuged at 700*g* for 10 min, the pellets were discarded and the supernatants centrifuged at 8000*g* for 10 min to precipitate mitochondria that were washed in the same conditions. Mitochondrial suspensions of about 15 mg protein/ml were either rapidly used to measure O_2 uptake, H_2O_2 production and mtNOS functional activity or frozen in liquid N_2 and kept at -80 °C for other determinations. Mitochondrial membranes (fragments) were obtained from twice frozen and thawed mitochondria homogenized each time by passage through a hypodermic needle. Mitochondrial protein was determined by the Folin reagent with bovine serum albumin as standard.

5. Tyrosine hydroxylase immunohistochemistry

Brain transversal sections were washed and incubated with mouse anti-tyrosine hydroxylase antibody (MAB5280, 1/1000) for 24 hours. The sections were incubated with biotinylated anti-mouse IgG (BA2001; 1/200) and with an antibody forming an avidin-peroxidase complex for reaction visualization by incubation with diaminobenzidine in 0.3% H_2O_2 [42]. High-resolution photos were obtained with a digital camera in a Nikon microscope DXM1200F Optiphot-2 and processed with Image-J software (National Institutes of Health, Bethesda, MD, USA).

6. Tissue and mitochondrial respiration

6.1. Tissue O_2 uptake

Striatum and frontal cortex were sectioned into about 1-mm tissue cubes and the O_2 uptake of 4–8 cubes was determined with a Clark electrode (Hansatech Instruments Ltd., Norfolk, UK) in a 1.5-ml chamber at 30 °C in air-saturated Krebs medium with 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.40 [24]. After 1-2 min, tissue O_2 uptake, linearly dependent on the number of tissue cubes, remained stable and was recorded for 2-3 min. Oxygen uptake was expressed in ng-at O/min $\times$ g tissue.

6.2. Mitochondrial O_2 uptake

This was determined with a Clark electrode in a 1.5 ml chamber at 30 °C in an air-saturated reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM KH_2PO_4/K_2HPO_4, 3 mM $MgCl_2$, pH 7.40, and 0.6-0.8 mg protein/ml. Respiration was determined with either 5 mM malate-5 mM glutamate or 10 mM succinate as substrates. State 3 active respiration was established by addition of 0.50 mM ADP [41]. Results were expressed as ng-at O/ min $\times$ mg protein and the respiratory control ratio (RC) was calculated as the ratio of state 3/state 4 respiration rates.

6.3. Biochemical and functional mtNOS activities

Nitric oxide production by biochemical mtNOS activity was determined by the oxyhemoglobin (HbO_2) oxidation assay, followed at the HbO_2 α-band at 37 °C [43] in a modification of the assay described by Ignarro et al. [27]. The HbO_2 α-band makes the determination in turbid suspensions possible by decreasing light scattering. The reaction medium consisted of 0.10 mM NADPH, 1.0 mM L-arginine, 1.0 mM $CaCl_2$, 2.0 μM Cu,Zn-superoxide dismutase (Cu,Zn-SOD), 0.1 μM catalase and 20 μM HbO_2 hem, 50 mM KH_2PO_4/K_2HPO_4, pH 5.80, and 0.4-0.6 mg mitochondrial protein/ml [44]. A diode array

spectrophotometer (8453 Agilent Corp., Palo Alto, CA, USA) was used with 577 nm as active wavelength with a reference wavelength at the isosbestic point of 591 nm ($\varepsilon_{577\text{-}591}$= 11.2 mM^{-1} cm^{-1}). NO production was calculated from the absorbance change inhibited by 2 mM L-NMMA, usually 92-96%, and expressed in nmol NO/min × mg protein.

Mitochondrial NOS functional activity was determined by the inhibition of mitochondrial O_2 consumption by endogenous NO production, as observed by Giulivi [8] and described by Valdez et al. [45]. The assay determines the difference between the state 3 rates of O_2 uptake, at maximal NO steady state concentrations in the presence of 1.0 mM L-arginine and 1.0 μM Cu,Zn-SOD, and at minimal NO steady state levels in the presence of 2.0 mM L-NAME and 20 μM oxyhemoglobin (HbO_2).

6.4. Respiratory complexes activities

The enzymatic activities of complexes I, I-III, II-III, and IV were determined spectrophotometrically at 30 °C using mitochondrial membranes [46]. Complex I-III (NADH-cytochrome *c* reductase) and complex II-III (succinate-cytochrome *c* reductase) activities were determined in 100 mM KH_2PO_4/K_2HPO_4, pH 7.40, with 0.20 mM NADH or 5.0 mM succinate as substrates, 0.10 mM cytochrome c^{3+} and 1.0 mM KCN at 550 nm (ε=19 mM^{-1} cm^{-1}) and expressed as nmol reduced cytochrome *c* / min × mg protein. Complex I activity was also determined as NADH-ferricyanide reductase at 420 nm in the same buffer with 0.20 mM NADH, 1 mM $K_3Fe(CN)_6$ (ε = 1.0 mM^{-1} cm^{-1}) and 1 mM KCN and expressed as nmol reduced ferricyanide/min × mg protein. Cytochrome oxidase (complex IV) was determined at 550 nm in the reaction medium used for complex I with 50 μM cytochrome c^{2+}, as the pseudo first order reaction constant (k')/mg protein and expressed as nmol oxidized cytochrome *c* at 20 μM cytochrome c^{2+}, that results in electron transfer rates similar to those of physiological respiration.

7. Reactive oxygen species and protein and lipid oxidation products

7.1. Superoxide anion and hydrogen peroxide production

The rates of $O_2^{\bullet-}$ and H_2O_2 production were determined in mitochondrial fragments at 30 °C [47]. Superoxide radical was determined spectrophotometrically following the reduction of 20 μM acetylated cytochrome c^{3+} at 550-540 nm (ε = 19 mM^{-1} cm^{-1}) [38]. Hydrogen peroxide was determined fluorometrically at 365 nm (excitation) and 450 nm (emission) with 0.5 μM horseradish peroxidase and 1.0 μM scopoletin. The reaction medium was 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl. pH 7.40, with 50 μM NADH as substrate and 0.05-0.10 mg protein/ml. For $O_2^{\bullet-}$ determinations, mitochondrial fragments were washed three times in 140 mM KCl, 20 mM Tris-HCl, pH 7.40, to eliminate endogenous SOD. The specificities for $O_2^{\bullet-}$ and H_2O_2 reaction were confirmed by 2 μM Cu,Zn-SOD or 2 μM catalase that inhibited the assays by 96-98%. SOD-sensitive and catalase-sensitive rates were expressed as nmol $O_2^{\bullet-}$ or H_2O_2/min × mg protein.

7.2. Tissue oxidative damage

The mitochondrial content of thiobarbituric acid-reactive substances (TBARS) and of protein carbonyl groups (>C=O) were determined as described [24,46]. Phospholipid peroxidation was expressed in nmol TBARS/mg protein and protein oxidation in nmol carbonyl group/mg protein.

8. Cytochrome oxidase activity and cytochrome content

8.1. Mitochondrial content

Tissue mitochondrial mass was determined from the ratio of cytochrome oxidase activities in homogenates and in mitochondria [24]. Cytochrome oxidase activity was determined in 100 mM KH_2PO_4/K_2HPO_4, pH 7.40, with 50 μM cytochrome c^{2+}. Cytochrome c^{2+} oxidation by complex IV was calculated as the pseudo-first-order reaction constant (k´) and expressed as min/mg mitochondrial protein or min/g tissue homogenate. The ratio

between the activity measured in homogenates (k´/g tissue) and in mitochondrial membranes (k´/mg protein) yields the mitochondrial mass per gram of tissue. Reduced cytochrome *c* was freshly prepared by reduction of cytochrome c^{3+} with $Na_2S_2O_4$ followed by Sephadex G-25 chromatography.

8.2. Mitochondrial cytochrome content

Cytochromes were determined in a diode array spectrophotometer by scanning between 650 and 500 nm the absorption difference between dithionite reduced and oxidized samples, at the following wavelengths and extinction coefficients: cytochromes *c* + c_1, 550-540 nm and 19 mM^{-1} cm^{-1}; cytochromes b_K + b_T, 562-575 nm and 22 mM^{-1} cm^{-1}; cytochromes *a* + a_3, 605-630 nm and 21 mM^{-1} cm^{-1} [49].

9. Statistics

Values are means ± SEM (n = 3). Striatum and frontal cortex from 5 rats were pooled for mitochondrial isolation. Differences were analyzed by one-way ANOVA and by Student-Newman-Keuls tests. Values of $p<0.05$ and $p<0.01$ were considered significant and highly significant.

RESULTS

1. Neurologic and locomotive tests

After 6 weeks of rotenone treatment, rats showed severe bradykinesia with a marked increase (6-fold) in the catalepsy test (Fig. 1A). Similarly, an important decrease (60%) was observed in spontaneous locomotive activity in the open field test (Fig. 1B).

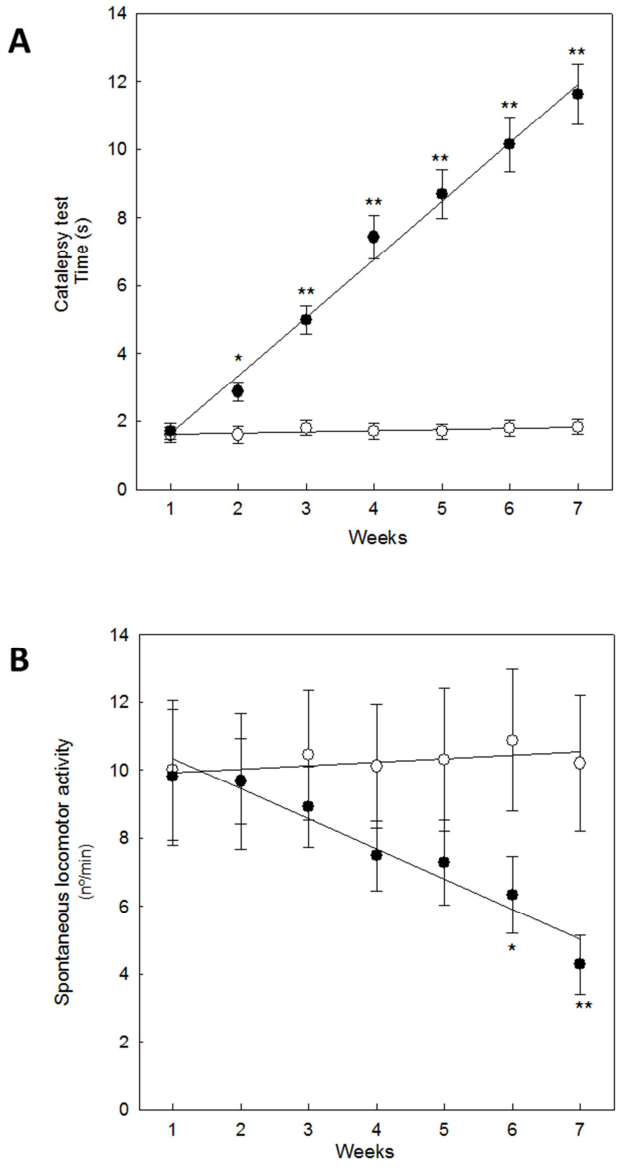


Figure 1. Effect of rotenone treatment on rat motor activity. (A) catalepsy test, and (B) open field spontaneous locomotor activity. Control rats (open circles) and rotenone-treated rats (closed circles). Rotenone-treated versus control, *p<0.05, **p<0.01.

2. Tyroxine hydroxylase histochemistry

Transversal brain sections showed a highly decreased tyrosine hydroxylase activity in 60 days rotenone-treated rats as compared with control animals (Fig. 2). The affected mid-brain area included the nigrostriatal pathway. Decreased tyrosine hydroxylase histochemistry indicated a marked cell death with loss of dopaminergic neurons. The loss was estimated at about 50% by spectrophotometric determination of film absorbance.

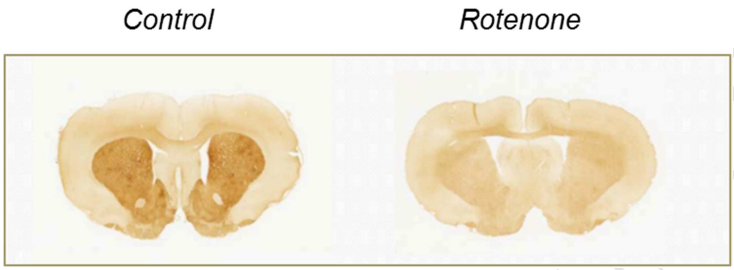


Figure 2. Tyrosine hydroxylase immunohistochemistry of brains from control and 60 days rotenone treated rats.

3. Striatum and frontal cortex tissue O_2 consumption

Striatum and frontal cortex exhibited linear respiratory rates that were decreased after 60 days of rotenone treatment by 35 and 10%, respectively, with intermediate values at 30 days (Table 1).

Table 1. Tissue O_2 uptake of striatum and frontal cortex in rotenone-treated and control rats

		Oxygen consumption (ng at O/min × g tissue)	
Brain tissue	**Control**	**Rotenone**	
		30 days	**60 days**
Striatum	896 ± 24 (100%)	744 ± 21** (83%)	582 ± 20*** (65%)
Frontal cortex	992 ± 23 (100%)	928 ± 22 (94%)	893 ± 22* (90%)

In parenthesis, respiration rates expressed as percentage of control values.

***p<0.001, **p<0.01, *p<0.05, rotenone-treated versus control rats.

4. Mitochondrial function

4.1. Mitochondrial O_2 consumption

Striatum and frontal cortex mitochondria of rotenone-treated rats showed a significantly decreased active respiration (state 3, with ATP synthesis) with malate-glutamate as substrate (Table 2). The inhibitions of O_2 uptake in striatum mitochondria were 16 % and 34 % after 30 and 60 days of rotenone treatment. Resting (state 4) respiration remained unchanged and respiratory control, the ratio state 3/state 4 of O_2 uptakes, declined according to the decreased state 3 respiratory rates. Frontal cortex mitochondria exhibited a similar, although less marked, respiratory inhibition that was only 16 % after 60 days of rotenone treatment.

In contrast, with succinate as substrate both striatum and frontal cortex state 3 O_2 uptake was not significantly modified.

Table 2. Oxygen uptake of striatum and frontal cortex mitochondria from rotenone-treated and control rats

	Oxygen consumption (ng-at O/min × mg protein)		
	Control	**Rotenone**	
		30 days	**60 days**
STRIATUM			
Malate-glutamate			
State 3	166 ± 9 (100%)	140 ± 8 (84%)	109 ± 7** (66%)
State 4	42 ± 3	40 ± 3	38 ± 3
Respiratory control	3.9 ± 0.3	3.5 ± 0.3	2.0 ± 0.3*
Succinate			
State 3	220 ± 12 (100%)	210 ± 11 (95%)	199 ± 9 (90%)
State 4	60 ± 4	58 ± 4	52 ± 4
Respiratory control	3.7 ± 0.3	3.6 ± 0.3	3.7 ± 0.3
FRONTAL CORTEX			
Malate-glutamate			
State 3	143 ± 8 (100%)	133 ± 8(93%)	120 ± 7* (84%)
State 4	27 ± 2	26 ± 2	25 ± 2
Respiratory control	5.3 ± 0.3	5.1 ± 0.3	4.8 ± 0.4
Succinate			
State 3	179 ± 9 (100%)	169 ± 9 (94%)	164 ± 3 (92%)
State 4	44 ± 3	44 ± 3	42 ± 3
Respiratory control	4.1 ± 0.3	3.8 ± 0.3	3.6 ± 0.3

In parenthesis, state 3 respiration as percentage of control values

**p<0.01, *p<0.05, rotenone-treated versus control rats

4.2. Activities of mtNOS and of respiratory complexes

Striatum mtNOS activity was decreased to 73% and to 40% after 30 and 60 days of rotenone treatment (Table 3). Moreover, complex I activity was also diminished to 57-43 % after 60 days of treatment, measured as NADH-cytochrome *c* reductase and as NADH-ferricyanide reductase, with intermediate effects after 30 days of treatment. Accordingly, similar marked declines of complex I and mtNOS activities were observed in striatum mitochondria after rotenone treatment. In addition, complex I activity was significantly reduced to 70 % in frontal cortex after 60 days of treatment.

In contrast, complexes II-III and IV activities were not significantly modified in either of the tissues studied.

Table 3. Activities of mtNOS and of respiratory complexes in striatum and frontal cortex mitochondria from control and rotenone-treated rats

	Mitochondrial activities		
	Control	**Rotenone**	
		30 days	**60 days**
STRIATUM			
mtNOS	0.48 ± 0.04 (100%)	0.35 ± 0.04 (73%)	0.19 ± 0.03** (40%)
Complex I	170 ± 11 (100%)	141 ± 10 (83%)	73 ± 6*** (43%)
Complex I-III	260 ± 12 (100%)	216 ± 12 (83%)	148 ± 10 (57%)
Complex II-III	119 ± 9 (100%)	117 ± 9 (98%)	115 ± 9 (97%)
Complex IV	150 ± 12 (100%)	122 ± 10 (81%)	116 ± 10 (77%)
FRONTAL CORTEX			
mtNOS	0.45 ± 0.04 (100%)	0.44 ± 0.04 (98%)	0.41 ± 0.03 (91%)
Complex I	207 ± 12 (100%)	200 ± 11 (97%)	145 ± 10* (70%)
Complex I-III	309 ± 15 (100%)	303 ± 14 (98%)	278 ± 12 (90%)
Complex II-III	121 ± 9 (100%)	119 ± 9 (99%)	118 ± 8 (98%)
Complex IV	202 ± 18 (100%)	178 ± 16 (88%)	164 ± 15 (81%)

In parenthesis, enzymatic activities expressed as percentages of control values.

mtNOS activity is given in nmol NO/min × mg protein. Activities of complexes I and II are given in nmol reduced cytochrome *c*/ min × mg protein. Complex IV activity is expressed as the pseudo first order constant (k´)/mg protein at 20 μM cytochrome *c*.

***p<0.001, **p<0.01, *p<0.05, rotenone-treated versus control rats

4.3. Functional activity of mtNOS in striatum mitochondria

Mitochondrial NOS functional activity was assayed through the inhibition of O_2 consumption produced by NO [45]. State 3 respiratory rates were determined at maximal (L-arginine and SOD added) and minimal (L-NAME and HbO_2 added) intramitochondrial NO concentration. The difference between the state 3 O_2 uptake in the presence of L-arginine and SOD (a) and in the presence of a NOS inhibitor and HbO_2 (b) indicates the mtNOS functional activity in the inhibition of cytochrome oxidase activity. Table 4 shows that supplementation of control striatum mitochondria with L-arginine and SOD, providing the mtNOS substrate and removing extramitochondrial $O_2^{\bullet-}$, decreased state 3 respiration, while the addition of L-NAME and HbO_2 to the reaction medium increased O_2 uptake. Thus, the mtNOS functional activity in control striatum mitochondria accounted for 14% of state 3 O_2 uptake, and decreased to 71% after 30 days of rotenone treatment and to 29% after 60 days of treatment. A remarkable agreement in the inhibitions of mtNOS activity determined by the biochemical and by the functional activity assays was observed (Fig. 3). Moreover, linear correlations were observed (Fig. 3) between complex I activity and mtNOS biochemical and functional activities (r^2 = 0.98 and 0.97) and between complex I activity and malate-glutamate supported state 3 O_2 uptake (r^2 = 0.98), measured in striatum mitochondria, indicating that the pattern observed for complex I activity decline is associated to mtNOS activity reduction and to the impairment of striatum mitochondrial respiration sustained by malate-glutamate.

Table 4. Functional activity of mtNOS in striatum mitochondria from rotenone-treated and control rats.

	Oxygen consumption (ng-at O/min × mg protein)		
	Control	**Rotenone**	
		30 days	**60 days**
State 3 (malate-glutamate)	166 ± 9 (100%)	140 ± 8 (84%)	109 ± 7 (66%)
[a] State 3 + L-arginine + SOD	158 ± 11	133 ± 9	113 ± 8
[b] State 3 + L-NAME + HbO_2	182 ± 12	150 ± 10	120 ± 9
[b-a] mtNOS functional activity	24 ± 2 (100%)	17 ± 2 (71%)	7 ± 2** (29%)

In parenthesis, percentage of the control values of state 3 respiration and of mtNOS functional activity. **p<0.01, rotenone-treated versus control rats

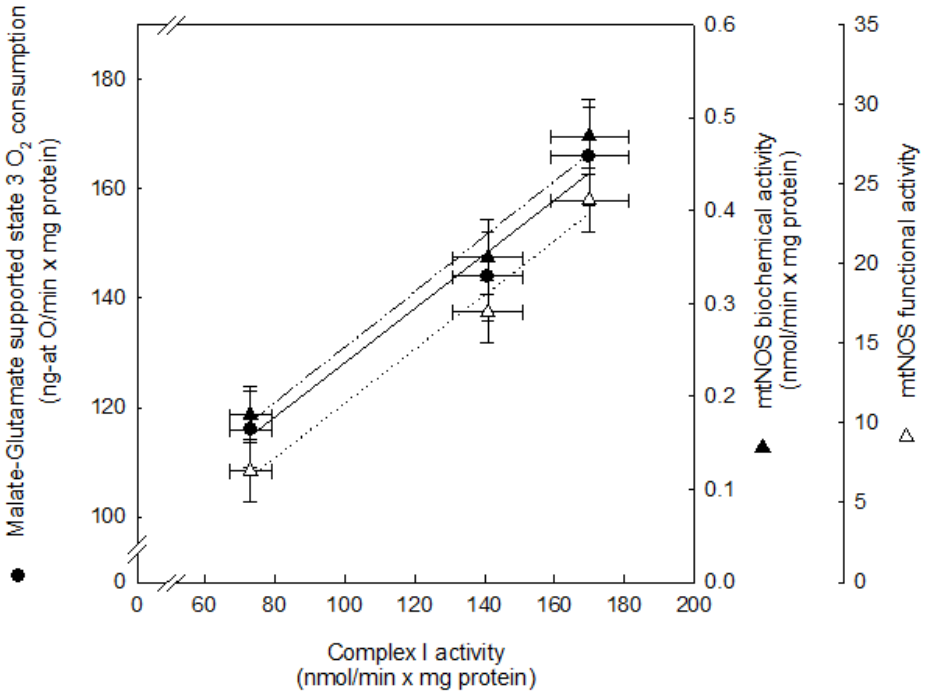


Figure 3. Linear correlations between mitochondrial complex I activity and malate-glutamate supported state 3 respiration (●) (r^2 = 0.97) and between complex I activity and mtNOS biochemical (▲) (r^2 = 0.98) and functional activities (Δ) (r^2 = 0.98).

On the other hand, no significant changes were observed in mtNOS functional activity in frontal cortex (data not shown), in agreement with no significantly changes observed in mtNOS biochemical activity (Table 3) in this brain area.

5. Mitochondrial $O_2^{\bullet-}$ and H_2O_2 production and products of oxidative damage

The $O_2^{\bullet-}$ and H_2O_2 production rates of striatum and frontal cortex mitochondria were increased (34-44%) after 60 days of rotenone-treatment, with intermediate values at 30 days (Table 5). The ratios 1.9-2.1 of $O_2^{\bullet-}$ over H_2O_2 rates respectively, indicate that $O_2^{\bullet-}$ is the stoichiometric precursor of mitochondrial H_2O_2.

Phospholipid peroxidation and protein oxidation were increased in striatum and frontal cortex mitochondria after 60 days of treatment (Table 5). Phospholipid peroxidation, determined as TBARS, increased 28-36%, and protein oxidation, determined as >C=O groups, was enhanced by 30-46% in frontal cortex and striatum mitochondria.

Table 5. Mitochondrial production of $O_2^{\bullet-}$ and of H_2O_2 and TBARS and protein carbonyls contents in striatum and frontal cortex mitochondria of control and rotenone-treated rats

	Control	Rotenone	
		30 days	60 days
STRIATUM			
$O_2^{\bullet-}$ production	1.50 ± 0.07	1.61 ± 0.07	2.01 ± 0.08**
H_2O_2 production	0.70 ± 0.03	0.82 ± 0.04	0.98 ± 0.04**
Phospholipid peroxidation	1.48 ± 0.05	1.74 ± 0.05*	2.02 ± 0.06***
Protein oxidation	26 ± 2	32 ± 3	38 ± 3*
FRONTAL CORTEX			
$O_2^{\bullet-}$ production	1.17 ± 0.06	1.21 ± 0.06	1.65 ± 0.08**
H_2O_2 production	0.57 ± 0.03	0.59 ± 0.03	0.82 ± 0.04**
Phospholipid peroxidation	1.39 ± 0.05	1.68 ± 0.06*	1.78 ± 0.06**
Protein oxidation	23 ± 2	26 ± 2	30 ± 2

$O_2^{\bullet-}$ and H_2O_2 productions are expressed as nmol/min × mg protein; phospholipid peroxidation as nmol TBARS/mg protein; and protein oxidation as nmol carbonyl group/ mg protein. ***p<0.001, **p<0.01, *p<0.05, rotenone-treated versus control rats

6. Tissue contents of mitochondria and cytochromes

Mitochondrial content was 24% decreased in striatum after 60 days of rotenone treatment (Table 6), in agreement with decreased tissue respiration and with impaired mitochondrial biogenesis, probably due to the time of rotenone treatment is about twice the time of brain mitochondrial turnover. The mitochondrial content in frontal cortex was not modified. Rotenone treatment did not affect cytochrome *b* and *c* contents in striatum and frontal cortex mitochondria whereas cytochrome aa_3 content was 31% lower in striatum

mitochondria after 60 days of treatment while it remained unchanged in frontal cortex (Table 6).

Table 6. Mitochondrial mass and cytochrome content in striatum and frontal cortex of rotenone-treated rats

	Control	Rotenone	
		30 days	60 days
MITOCHONDRIAL CONTENT			
STRIATUM			
[a] Homogenate	908 ± 80	716 ± 72	534 ± 35*
[b] Mitochondria	75 ± 8	61 ± 5	58 ± 5
[a/b] Mitochondrial mass	12.1 ± 0.3	11.8 ± 0.3	9.2 ± 0.3***
FONTAL CORTEX			
[a] Homogenate	1364 ± 95	1187 ± 90	1042 ± 90
[b] Mitochondria	101 ± 10	89 ± 9	82 ± 12
[a/b] Mitochondrial mass	13.5 ± 0.4	13.3 ± 0.4	12.7 ± 0.3
CYTOCHROME CONTENT			
STRIATUM			
Cytochromes c + c_1	0.22 ± 0.02	0.19 ± 0.02	0.15 ± 0.02
Cytochromes b_K +b_T	0.17 ± 0.02	0.17 ± 0.02	0.15 ± 0.02
Cytochromes a + a_3	0.16 ± 0.02	0.13 ± 0.02	0.11 ± 0.02
FRONTAL CORTEX			
Cytochromes c + c_1	0.19 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
Cytochromes b_K +b_T	0.18 ± 0.02	0.17 ± 0.02	0.16 ± 0.02
Cytochromes a + a_3	0.16 ± 0.02	0.16 ± 0.02	0.16 ± 0.02

DISCUSSION

Rotenone is a high affinity stoichiometric inhibitor of mitochondrial complex I fitting in a crevice of complex I structure. In brain, heart and liver mitochondria full inhibitions are observed at 0.10 nmol rotenone/mg protein [23,50]. It has been described that the chronic intraperitoneal rotenone model is able to reproduce the two pathological hallmarks of PD, *i.e.* the degeneration of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies in the surviving substantia nigra neurons as well as other regions of the central and peripheral nervous system, in addition to motor deficits, extranigral pathology and certain parkinsonian non-motor symptoms [36,38,39]. Moreover, it has been observed a good correlation between the chronic intraperitoneal rotenone-induced motor symptoms and the loss of tyrosine hydroxylase-positive neurons or dopamine depletion in the nigrostriatal pathway, as well as the non-motor symptoms and clinical state [51]. From the mitochondrial point of view, a complex I dysfunction has been reported in PD patients that to contribute to neurodegeneration [32,38,51,52]. Due to rotenone is able to penetrate all cells and mitochondria, chronic intraperitoneal rotenone model is useful to study the mitochondrial bioenergetics profile and for the understanding the brain mitochondrial impairments in human PD. Consequently, this *in vivo* model reproduces the inhibition of complex I by rotenone that has been observed *in vitro,* being this one of the pathognomonic characteristics of PD.

In the present article, after 60 days of rotenone treatment, decreased striatum (34%) and frontal cortex (16%) state 3 malate-glutamate sustained respiration (Table 2) were observed. This impairment in mitochondrial O_2 consumption was reflected in tissue respiration, since comparable O_2 uptake declines, in rat striatum (35%) and frontal cortex (10%), were observed (Table 1). In sections of perfused tissues and organs, O_2 consumption rate depends is mainly accounted for the sum of the O_2 uptakes of mitochondria respiring in state 3 and of mitochondria respiring in state 4. The decays in O_2 uptake after 60 days of rotenone treatment were higher in striatum than in frontal cortex.

Concerning respiratory complexes activities, only complex I activity was reduced in striatum and frontal cortex after 60 days of rotenone treatment (Table 3). These results are in agreement with those reported by Telford et al [53]. These authors have shown using metabolic control analysis that complex I is the component of the electron transfer chain which exerts a high level of control over synaptosomal bioenergetics. Consequently, complex I deficiencies, as for instance the one observed in neurodegenerative disorders, are sufficient to compromise O_2 consumption. Striatum mitochondria showed not only 57% decreased complex I activity but also 60% decreased mtNOS activity (Table 3). Moreover, the functional activity of mtNOS, *i.e.* the inhibition of cytochrome oxidase and therefore O_2 uptake by endogenous NO, was markedly decreased after 60 days of rotenone treatment, with an intermediate value after 30 days of treatment (Table 4).

Striatum and frontal cortex mitochondria showed increased production rates of $O_2^{\bullet-}$ (34% and 41%) and H_2O_2 (40% and 44%), after 60 days of treatment (Table 5). To note, although the enhancement percentages in $O_2^{\bullet-}$ and H_2O_2 productions in striatum are similar to the ones observed in frontal cortex, the net rate values of those productions are lower in this latter tissue. This fact is in agreement with the increases observed in phospholipid peroxidation and protein oxidation products in striatum (36% and 46%) and in frontal cortex (28% and 30%) (Table 5). In addition, considering also the mitochondrial respiratory dysfunction, in terms of state 3 O_2 consumption sustained by malate-glutamate (Table 2) and complex I and mtNOS activities (Table 3), the “damage pattern” was lower in frontal cortex than in striatum, suggesting that frontal cortex mitochondria display a different response to changes in redox status, probably due to the antioxidant defences profile. It is important to consider that the degree of damage depends, among others, on $O_2^{\bullet-}$ steady state concentration, *i.e,* not only on $O_2^{\bullet-}$ production rate but also on $O_2^{\bullet-}$ decomposition, which mainly occurs through SOD-dismutation reaction and the reaction between $O_2^{\bullet-}$ and NO to yield $ONOO^-$. Despite of the overall changes were more markedly in striatum than in frontal cortex, both cerebral areas from rotenone-treated rats for 60 days exhibit a complex

I syndrome [5], similar to the one observed in frontal cortex mitochondria from PD patients [54].

Dysfunction of complex I is usually accompanied by changes in mtNOS activity, increases in H_2O_2 and $ONOO^-$ production rates and oxidative and/or nitrosative damage [5, 11,12,18-21]. The decline in complex I and in mtNOS activities is consistent with the two enzymes structurally contiguous and functionally associated [10-13]. Poderoso's group [10] showed that not only complex IV but also complex I proteins immunoprecipitate with mtNOS, suggesting a molecular interaction among mtNOS and complexes I and IV. Furthermore, the functional association between complex I and mtNOS agrees with the observations by Parihar et al. [11] and our group [55] who sustained that mtNOS is structurally adjacent to complex I. Moreover, data from our laboratory [13] have shown that heart mitochondrial inside-out particles produce NO supported by succinate-dependent reversed electron flow in the respiratory chain, emphasizing the notion that mtNOS and complex I proteins are functionally associated and contiguously located. Therefore, changes in complex I structure (~600 kDa) also affects adjacent mtNOS (130 kDa) conformation. It was speculated that the functional association between mtNOS and complex I is essential for preserving high rates of mitochondrial NO production and of mitochondrial biogenesis by the NO and cGMP dependent pathway [23,31]. The observations agree with the effects of rotenone in decreasing complex I activity and ATP levels with an over-expression of bcl-2 in striatum [56-57], and with complex I inhibition and increased phospholipid peroxidation in striatum bodies and frontal cortex [57].

The development of complex I syndrome in rotenone treated rats is understood as a self-sustained process, started by rotenone binding with increased complex I auto-oxidation and enhanced $O_2^{\bullet -}$ production [12]. This sustains a free-radical mediated process including: (a) the Haber-Weiss chemistry of $HO^{\bullet}$ production (eqs. 1-2) including the

$$Fe^{3+}(Cu^{2+}) + O_2^{\bullet -} \longrightarrow Fe^{2+}(Cu^{+}) + O_2 \qquad \text{eq. 1}$$

$$Fe^{2+}(Cu^{+}) + H_2O_2 \longrightarrow Fe^{3+}(Cu^{2+}) + OH^{-} + HO^{\bullet} \qquad \text{eq. 2}$$

the Fenton reaction (eq. 2) [58,59]; (b) the lipid peroxidation chain reaction with the generation of the reactive peroxyl radical ($ROO^{\bullet}$); (c) the reactions of the lipid peroxidation produced aldehydes (4-hydroxynonenal and malonaldehyde) with the amino groups of complex I proteins; and (d) the nitration of complex I proteins by the increased formation of peroxynitrite ($ONOO^-$), which is generated by the reaction of $O_2^{\bullet-}$ and NO at the vicinity of complex I active center where $O_2^{\bullet-}$ is produced and encounters the highly diffusive NO [60]. Generation of $ONOO^-$ seems to account for the main part of cell toxicity in rat brain. To note, even in physiopathological situations in which NO production is reduced, mitochondrial $ONOO^-$ production rate may be increased if the steady-state concentration of $O_2^{\bullet-}$ is increased, with the consequent oxidation of biomolecules or modification of proteins by nitration, because mitochondrial $ONOO^-$ generation is mainly driven by O_2^- steady-state concentration rather than by NO steady-state concentration [61]. Thus, these processes lead to complex I partial denaturalization, with polypeptide cross-linking, loss of native non-covalent bonds and increased auto-oxidation rates. These facts have been proposed as the pathogenic mechanism for brain neurodegenerative diseases [17, 24, 46, 54, 62-64]. Interestingly, signaling from mitochondria seems to regulate cell metabolism and the expression of the nuclear-encoded components of the mitochondrial respiratory chain, a mechanism impaired in PD [63]. Indeed, immunoblotting of striatum mitochondria from PD patients showed that the expression levels of the 30-, 25- and 24-kDa subunits of complex I were moderately to markedly decreased [64]. In rat striatum, complex I syndrome was accompanied by a decreased tissue mitochondrial content (Table 6) that worsens the mitochondrial dysfunction. Dopaminergic neurons are more sensitive to rotenone poisoning than other brain cells; likely because they are subjected to a basal oxidative stress by a high endogenous $O_2^{\bullet-}$ production in the autoxidation of catecholamines and dopamine. The reaction of NO and quinols produces semiquinones [65]. Interestingly, dopaminergic neurons in culture exposed to rotenone imitate some features of PD, showing α-synuclein, Lewy bodies and enhanced apoptosis [66].

It has been observed that complex I syndrome is always associated with oxidative stress. The concept of oxidative stress was originally described as an unbalance between oxidant production and antioxidant defense [67,68] that was immediately accepted and successfully applied to organelles, cells, tissues and whole organisms. Jones and Sies extended the classical conception of oxidative stress to the “redox code” concept, where the increased oxidative reactions disrupt redox signaling and control, leading to cellular damage [69]. A high reduction of thiol groups (–SH) in GSH and in low molecular weight thiols is essential for keeping the intracellular reductive state that supports cell metabolism, signaling and regulation.

There was an early recognition of increased lipid peroxidation [70] and of deficient complex I activity [52] in the *substantia nigra* of human PD patients. At present, both alterations are classic concepts of the biochemical alterations of dopaminergic neurons in PD. The present report shows that decreased mitochondrial NO production is another central alteration in PD. Microscopic examination of PD brains shows degeneration and death of dopaminergic neurons with Lewy bodies and α-synuclein in *substantia nigra,* medial temporal and limbic nuclei, and frontal cortex [32-33].

It is clear that the complex I syndrome observed in striatum and frontal cortex in rotenone-treated rats corresponds to a situation of oxidative stress and damage with increased rates of harmful free-radical mediated reactions. These reactions and processes are likely to occur in human PD, affording a pathogenic mechanism. Thus, despite of its limitations, chronic intraperitoneal rotenone model may considered to be sufficiently similar to human disease and it is useful for the understanding of the brain mitochondrial impairments in PD, as well as to evaluate and select drug candidates that affect these processes to support their progression into clinical trials. For instance, it is likely that pharmacologic interventions as supplementation with exogenous antioxidants as vitamin E and ascorbic acid would improve PD patient’s health.

AUTHORS CONTRIBUTION

Ana Navarro conceived the whole research project and designed and supervised the experiments performed by Manuel J. Bández and José María López-Cepero, who collected the original data. Laura B. Valdez and Tamara Zaobornyj drafted the paper with the experimental results and the final version was assembled by Alberto Boveris.

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COMPETING INTERESTS

The authors declare that there are no competing interests associated with this manuscript.

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HIGHLIGHTS

- Striatum and cortex mitochondria of rotenone-treated rats exhibit “complex I syndrome”
- Mitochondria show decreased O_2 uptake -with NAD-dependent substrates- and NO production
- Mitochondria show increased $O_2^{\bullet-}$, H_2O_2, lipid peroxidation and protein oxidation
- Rotenone-treated rats show decreased striatum mitochondrial mass
- Mitochondrial complex I is highly sensitive to inactivation by oxidative reactions