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Effects of rotenone and pyridaben on complex I electron transfer and on mitochondrial nitric oxide synthase functional activity

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Abstract Rotenone and pyridaben were tested on activities and properties of rat brain mitochondria determining Ki (inhibitor concentration at half maximal inhibition) and Imax (% of inhibition at maximal inhibitor concentration). The assayed activities were complexes I, II and IV, respiration in states 3, 3u (uncoupled) and 4, biochemical and functional activities of mitochondrial nitric oxide synthase (mtNOS), and inner membrane potential. Selective inhibitions of complex I activity, mitochondrial respiration and membrane potential with malate-glutamate as substrate were observed, with a Ki of 0.28–0.36 nmol inhibitor/mg of mitochondrial protein. Functional mtNOS activity was half-inhibited at 0.70–0.74 nmol inhibitor/mg protein in state 3 mitochondria and at 2.52–2.98 nmol inhibitor/mg protein in state 3u mitochondria. This fact is interpreted as an indication of mtNOS being structurally adjacent to complex I with an intermolecular mtNOS-complex I hydrophobic bonding that is stronger at high $\Delta\psi$ and weaker at low $\Delta\psi$.

Keywords mtNOS · Complex I-mtNOS interactions · Complex I conformational changes · Rotenone · Pyridaben

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Introduction

Mitochondrial complex I (NADH-ubiquinone reductase) is the obligatory pathway for the oxidation of NADH in aerobic organisms, comprising bacteria, higher plants and animals. Complex I is composed of 46 polypeptide chains and has a L-shaped structure, with the longer arm constituted by hydrophobic integral membrane polypeptides, the membrane domain, and the shorter arm as the hydrophilic domain extending into the mitochondrial matrix and carrying the NADH-active center (Carroll et al. 2003; Hirst et al. 2003). The two arms of the L-shaped complex I have a separated genetic origin, independent assembly and contribute differently to electron transfer and energy transduction (Hofhaus et al. 1991). Complex I has 14 central subunits, present from the bacterial to the mitochondrial versions of the enzyme, that carry the redox-active centers essential for electron transfer: FMN and 8-9 iron-sulfur clusters (Ohnishi 1998; Hinchliffe and Sazanov 2005). Mitochondrial complex I contains up to 32 accessory hydrophobic subunits, likely involved in the structural assembly of the multiprotein complex and that confer stability to the enzyme and provide for enzyme regulation (Abdrakhmanova et al. 2004; Dudkina et al. 2005; Brandt 2006). The FMN-containing polypeptide is in the hydrophilic arm of the enzyme that is located in the mitochondrial matrix and serves as electron acceptor for NADH. The FMN-containing polypeptide is adjacent to the polypeptides that contain the redox-active iron-sulfur centers and that provide a chain of electron transfer between FMN and ubiquinone (Walker 1992). The different genetic and evolutionary origin of complex I is reflected in its 3 functional modules: the electron input N module that oxidizes NADH, the P module that pumps protons across

the membrane, and the electron output Q module that reduces ubiquinone (Brandt 2006).

The respiration of isolated mitochondria in state 3 with malate-glutamate or succinate as substrates is enhanced by supplementation with NOS competitive inhibitors, such as NAME, L-NMMA, (Giulivi et al. 1998a) or with chlorpromazine (Lores-Arnaiz et al. 2004) and inhibited by addition of arginine (Giulivi 1998; Giulivi et al. 1998a; Valdez and Boveris 2007; Navarro and Boveris 2007). The effects are explained by the NO/O_2 competitive inhibition of cytochrome oxidase (Brown and Cooper 1994; Cleeter et al. 1994; Antunes et al. 2004) by the NO continuously produced by mitochondrial nitric oxide synthase (mtNOS). This effect evolved to an assay for the *in situ* activity of mtNOS in isolated mitochondria, named "mtNOS functional activity" (Valdez et al. 2005). The assay consists in determining state 3 respiratory rates in two parallel mitochondrial samples. The first sample, at "minimal NO level", is added with a NOS inhibitor, such as L-NAME or L-NMMA, and oxyhemoglobin (HbO_2), a condition in which an instantaneous increase in O_2 uptake is observed. The second sample, at "maximal NO level", is added with arginine and SOD and shows an immediate decrease in O_2 uptake. The sum of the effects (stimulation and inhibition), usually accounts for 30–35 % of state 3 respiration and corresponds to 200–260 nM NO in the intramitochondrial matrix, according to the Antunes *et al*. computational model and considering that the determinations are made with the O_2 electrode at about 150 μM O_2 (Antunes et al. 2004). Such an intramitochondrial NO level agrees with the 200 nM NO calculated for heart and liver mitochondria in state 3 (Valdez et al. 2005). In the present study, the determination of mtNOS functional activity provides an experimental approach to explore the physical interactions of mtNOS and complex I.

Rotenone and pyridaben are selective and stoichiometric inhibitors of complex I (Gomez et al. 2007). Rotenone, (2R,6aS,12aS)-1,2,6,6ª,12,12ª- hexahidro-2-isoprenil-8,9-dimetoxi-cromeno-[3,4-*b*]-furo-[2,3-*h*]-cromen-6-one) is a natural isoflavonoid produced by plants of the *Leguminosae* family, long ago recognized as a fish poison and currently used as pesticide in gardens and to kill fish in lakes and reservoirs. In mitochondrial studies, rotenone is the classic specific and stoichiometric inhibitor of complex I (Chance et al. 1963; Oberg 1964). Pyridaben (2-*tert*-butyl-5-(4-*tert*-butylbenzylthio)-4-chloropyridazin-3(2*H*)-one) is a synthetic pyridazinone that is also used as a pesticide and that in this study provides the second complex I inhibitor (Degli 1998; Hollingworth 2001).

Materials and methods

Animals Male Wistar rats 3 months old were used. The animals were siblings grown at the Department of Experimental Animals of the University of Cadiz, housed in groups of five animals at 22±2 °C with 12 h/12 h light/dark cycles and with full access to water and food. Animal experiments were carried out in accordance with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society, the Guidelines of the European Union Council (86/609/CEE), and the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals.

Isolation of mitochondria and preparation of mitochondrial membranes Rat brain mitochondria were isolated from the whole organ homogenized in 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA and 10 mM Tris-HCl, pH 7.40, at a ratio of 9 ml of homogenization medium/g of tissue in a small Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700*g* for 10 min and the supernatant at 8000*g* for 10 min to precipitate mitochondria that were washed in the same conditions (Boveris et al. 1999; Navarro et al. 2002; Navarro and Boveris 2004; Navarro et al. 2004). Mitochondrial suspensions prepared at about 20 mg protein/ml were used immediately after isolation for the determination of oxygen uptake or frozen in liquid N_2 and kept at −80 ° C for other determinations. Mitochondrial membranes (mitochondrial fragments) were obtained from mitochondria that were twice frozen and thawed and homogenized each time by passage through a tuberculin needle. The procedure yielded a preparation of mitochondrial membranes with 0.18–0.22 nmol cytochrome aa_3/mg protein. The protein content of the samples was determined using the Folin reagent and bovine serum albumin as standard (Navarro et al. 2007).

Incubation of mitochondria with rotenone or pyridaben Brain mitochondria and mitochondrial membranes were incubated during 3 min at 4 °C with 0–10 μM rotenone or pyridaben before the determinations.

Mitochondrial electron transfer activities The enzymatic activities of complexes I, II, and IV were determined spectrophotometrically in mitochondrial membranes at 30 °C. For complex I (assayed as NADH-cytochrome *c* reductase) and complex II (assayed as succinate-cytochrome *c* reductase) activities, mitochondrial membranes were suspended in 100 mM phosphate buffer (pH=7.40), added with 0.20 mM NADH or 5.0 mM succinate as substrates, 0.10 mM cytochrome c^{3+} and 1.0 mM KCN and the activities were determined at 550 nm (ε= 19 $mM^{-1}cm^{-1}$) and expressed as nmol cytochrome *c* reduced/min×mg protein. Cytochrome oxidase (complex IV) was determined in the same reaction medium with 50 μM cytochrome c^{2+}, prepared by reduction with

ascorbate and filtration through a Sephadex G25 column. The rate of cytochrome *c* oxidation was calculated as the first order reaction constant (*k'*)/mg protein and expressed as nmol cytochrome c^{2+} oxidized at 10 μM cytochrome *c*, which gives electron transfer rates of the order of physiological respiration (Navarro et al. 2002; Navarro and Boveris 2004).

Mitochondrial oxygen uptake and mtNOS functional activity The O_2 uptake of rat brain mitochondria (0.6–0.8 mg/ml) was determined with a Clark electrode in a 1.5 ml chamber at 30 °C in an air-saturated reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM phosphate, 3 mM $MgCl_2$ (pH=7.40). Respiratory rates were determined with 5 mM malate-5 mM glutamate or 10 mM succinate as substrates, and state 3 active respiration was established by adding 0.50 mM ADP. The mtNOS functional activity was determined by the difference between the rates of O_2 uptake at maximal level of NO (by addition of 0.20 mM L-arginine and 1 μM superoxide dismutase (Cu, Zn-SOD) and a minimal level of NO (by addition of 2 mM L-NAME and 20 μM HbO_2) (Navarro et al. 2002; Navarro and Boveris 2004).

Spectrophotometric determination of biochemical mtNOS activity The production of NO by mitochondrial membranes was determined by the HbO_2 oxidation assay at 30 °C (Boveris et al. 2002). The reaction medium consisted of 0.10 mM NADPH, 0.20 mM arginine, 1.0 mM $CaCl_2$, 4.0 μM Cu,Zn-SOD, 0.10 μM catalase and 25 μM HbO_2 heme in 50 mM phosphate, pH 5.80, and 0.5–0.7 mg mitochondrial protein/ml. A diode array spectrophotometer (model 8453 Agilent Corp., Palo Alto, California, USA) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic 591 nm ($\varepsilon_{577-591}$=11.2 $mM^{-1}cm^{-1}$). Production of NO was calculated from the absorbance change that was inhibited by 2 mM N^G-methyl-L-arginine, usually 92–96%, and expressed in nmol NO/min×mg protein respiration (Boveris et al. 2002; Navarro and Boveris 2004).

Mitochondrial membrane potential (Δψ) Mitochondrial membrane potential was determined by measuring rhodamine 123 (Rh-123) fluorescence (Emaus et al. 1986; Scaduto and Grotyohann 1999; Baracca et al. 2003; Boveris et al. 2006) with a Hitachi F-2500 spectrofluorometer at 503 nm→527 nm (excitation→emission) at 30 °C. Rat brain mitochondria (0.5–0.7 mg/ml) were suspended in 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM phosphate and 4 mM $MgCl_2$ (pH=7.40) and supplemented with 0.1 μM Rh-123, 5 mM malate-5 mM glutamate, 10 mM succinate, 0.5 mM ADP, 1 μM FCCP, and 0–10 μM rotenone or pyridaben. The fluorescence of Rh-123 was recorded before and after addition of mitochondria, followed through the state 4-state 3 transition and after that, the inhibitor was added. After stabilization, fluorescence was recorded and mitochondria were centrifuged at 8000 rpm for 5 min and supernatant fluorescence was measured (Boveris et al. 2006). The fluorescence corresponding to state 3u (FCCP) sample was subtracted from all fluorescence values. These fluorescence readings, that directly indicate the amount of Rh-123 internalized into mitochondria, are expressed in arbitrary units as a percentage of the Rh123 accumulated in state 3.

Statistics and data processing All determinations were made in duplicates and the data was processed for determination of the kinetic parameters (Ki and Imax).

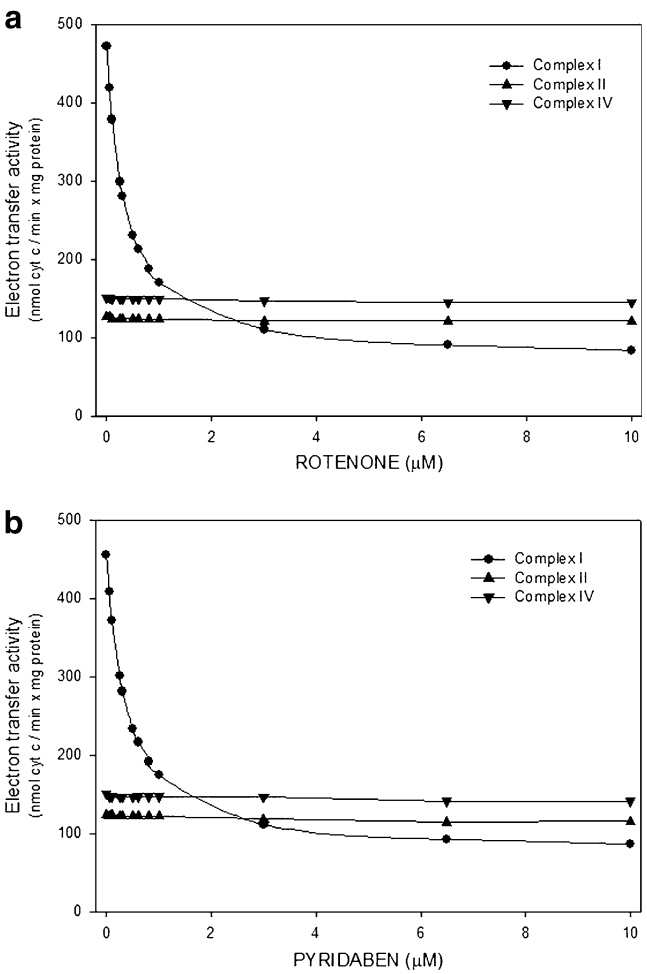

Fig. 1 Effects of rotenone (**a**) and pyridaben (**b**) on the activities of complex I (as NADH-cytochrome c reductase), of complex II (as succinate-cytochrome c reductase), and of complex IV (cytochrome oxidase) in rat brain mitochondrial membranes

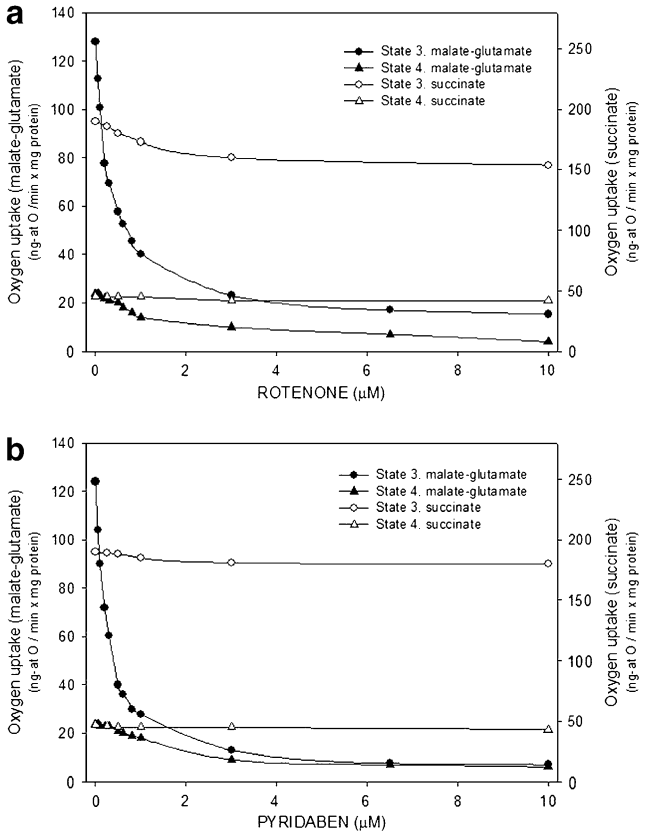

Fig. 2 Effects of rotenone (**a**) and pyridaben (**b**) on the respiration of rat brain mitochondria in metabolic states 4 and 3 with malate-glutamate or succinate as substrate

Results

The addition of either rotenone or pyridaben in the range of 0.05–10 μM to rat brain mitochondrial membranes showed a marked inhibition (84–85%) of complex I activity without significant change in the activities of complexes II and IV (Fig. 1). The inhibition of complex I is considered in terms of the chemical equilibrium: E+I ↔ EI, where E is complex I *in situ* in the mitochondrial inner membrane, I is the inhibitor (rotenone or pyridaben) and EI is the association of complex I and inhibitor, that does not have enzymatic activity.

The plots of enzyme activity (Fig. 1), oxygen uptake (Figs. 2, 3 and 4) or membrane potential (Fig. 6) as a function of inhibitor concentration show a descendant hyperbolic dependence that by double reciprocal plots (not shown) give Ki and Imax values. The first value (Ki) is the inhibitor concentration that gives 50% of the maximal inhibition and also 1/Keq of the given chemical reaction, and is expressed in relation to mg of mitochondrial protein, considering a 1:1 stoichiometric binding between inhibitor and complex I. The second value (Imax) is the maximal inhibition at infinite inhibitor concentration expressed as percentage of the enzymatic activity without inhibitor.

The Ki values for rotenone and pyridaben inhibitions of complex I activity, were 0.33 and 0.36 nmol/mg protein, respectively. The Imax for the two inhibitors on complex I activity were similar at 84–85 % (Table 1).

Rotenone and pyridaben also markedly (Imax=95–94%) and specifically inhibited the state 3 O_2 uptake of rat brain mitochondria with malate-glutamate as substrate with a Ki of 0.33 and 0.35 nmol/mg protein, respectively (Fig. 2 and Table 1). The inhibitory effect depended of metabolic state and substrate: a much less marked effect was observed in state 4 mitochondria, and respiration was not affected when succinate was the substrate (Fig. 2).

The functional activity of mtNOS in brain mitochondria and its sensitivity to rotenone and pyridaben was determined in states 3 (with ADP) and 3u (with FCCP) using malate-gutamate or succinate as substrates (Figs. 3 and 4). It is worth noting that mtNOS functional activity was easily and reliably determined in rat brain mitochondria in states 3 and 3u. With malate-glutamate, the rates in the absence of inhibitor were 31–33 ng-at O/min×mg protein (31–33% of state 3 or 3u respiration) and with succinate, the rates were 55–58 ng-at O/min × mg protein (29–31% of state 3 and 3u respiration), as it can be seen in the data points at 0 inhibitor in

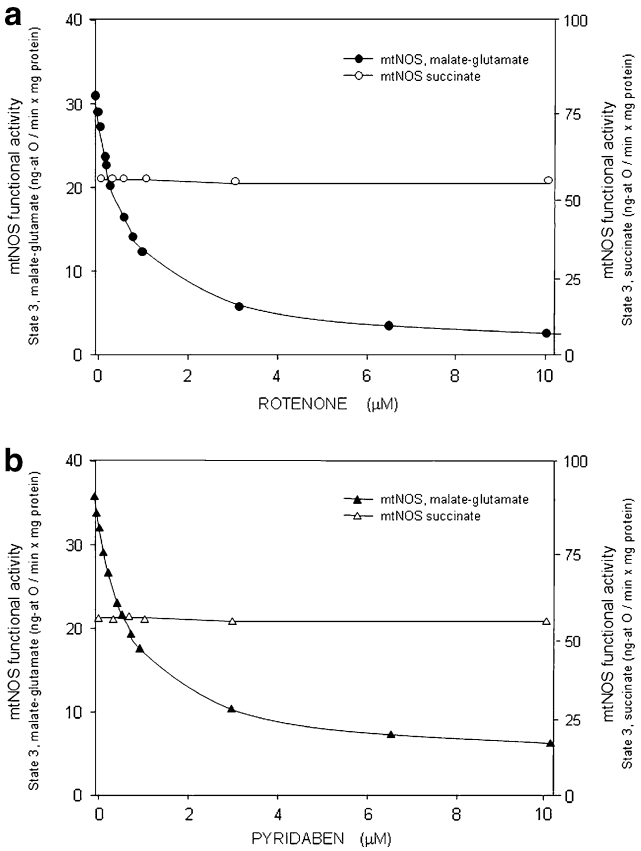

Fig. 3 Effects of rotenone (**a**) and pyridaben (**b**) on the mtNOS functional activity of state 3 rat brain mitochondria with malate-glutamate or succinate as substrate

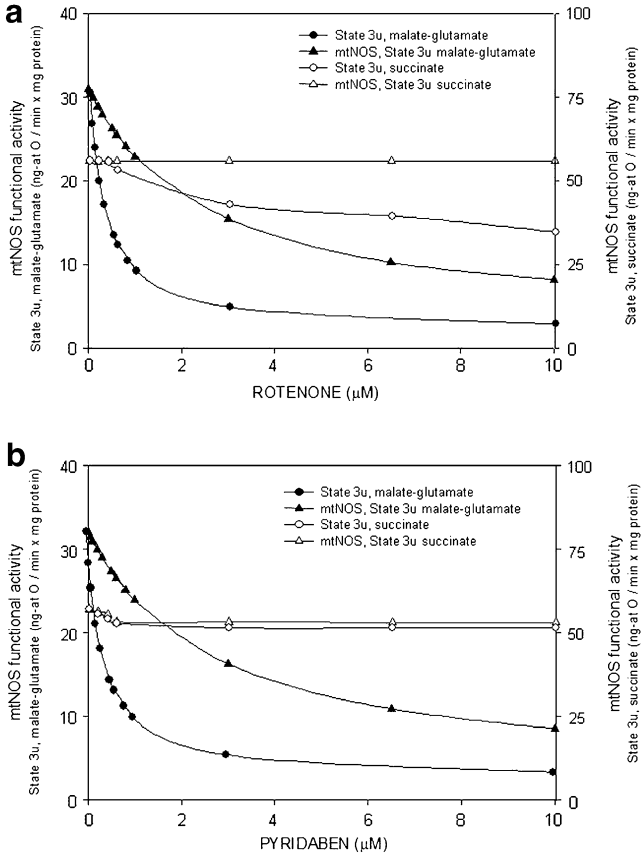

Fig. 4 Effects of rotenone (**a**) and pyridaben (**b**) on the respiration and mtNOS functional activity of state 3u (with 1 μM FCCP) rat brain mitochondria with either malate-glutamate or succinate as substrate. The rates of O_2 uptake in mtNOS functional activity and in state 3u have been normalized to illustrate the difference in Ki

Figs. 3 and 4. State 4 respiration was by far less sensitive (not shown) with levels of mtNOS functional activity of 4–10%, in full agreement with the computational model of NO effects on cytochrome oxidase activity (Antunes et al. 2007).

The mtNOS functional activity of rat brain mitochondria with malate-glutamate as substrate was inhibited by rotenone and pyridaben with an Imax of 92–96% in states 3 and 3u (Table 1). The Ki values for these effects were in the range of 0.70–0.74 for state 3 and of 2.52–2.98 for state 3u, in nmol of inhibitor/mg of protein (Table 1). This marked difference is explained by the much higher NO production in state 3 ($\Delta\Psi$=170–180 mV) than in state 3u ($\Delta\Psi$=0 mV) (Boveris et al. 2006). It needs to be stated that mtNOS functional activity is the difference between the rates of O_2 uptake between two conditions: (a) with NAME and HbO_2 added, and (b) with arginine and SOD added, so the direct inhibition of respiration by the complex I inhibitors in the two conditions are cancelled in the subtraction. The effect of rotenone and pyridaben in inhibiting mtNOS functional activity with NAD-linked substrates is selective, the same activity was not affected when succinate was the substrate in mitochondrial states 4, 3, or 3u.

Rotenone and pyridaben were ineffective as direct inhibitors of mtNOS biochemical activity, as it is determined using a classic reaction medium containing NOS substrates and cofactors (Boveris et al. 2002; Navarro and Boveris 2004) (Fig. 5).

The effect of rotenone and pyridaben on mitochondrial membrane potential was assayed by determining the mitochondrial accumulation of Rh-123; the process was inhibited up to 60 % with malate-glutamate but was almost unaffected with succinate (Fig. 6). The Ki values 0.29 and 0.32 μM for rotenone and pyridaben are similar to the others that reflect complex I inhibition (Table 1).

A summary of the rotenone and pyridaben effects is given in Table 1 as a comparative support for the hypothesis of a structural adjacency between mtNOS and complex I. A solid state model of the respiratory chain showing the structural integration of mtNOS with complexes I and IV is given in Fig. 7.

Discussion

Several reports have indicated that complexes I, III and IV interact to form supercomplexes with a defined stoichiometry

Table 1 Kinetic parameters of the inhibition by rotenone and pyridaben of complex I activity and related mitochondrial functions in rat brain mitochondria

Preparation/assay	Rotenone		Pyridaben	
	Ki (nmol/mg protein)	Imax (%)	Ki (nmol/mg protein)	Imax (%)
Mitochondrial fragments; complex I activity	0.33	85	0.36	84
Mitochondria; state 3 respiration (malate-glutamate)	0.33	95	0.35	94
Mitochondria, state 3u respiration (malate-glutamate)	0.34	94	0.34	92
Mitochondria; mtNOS functional activity, state 3 (malate-glutamate)	0.70	95	0.74	96
Mitochondria; mtNOS functional activity, state 3u (malate-glutamate)	2.52	92	2.98	93
Mitochondria; membrane potential ($\Delta\Psi$)	0.28	56	0.32	54

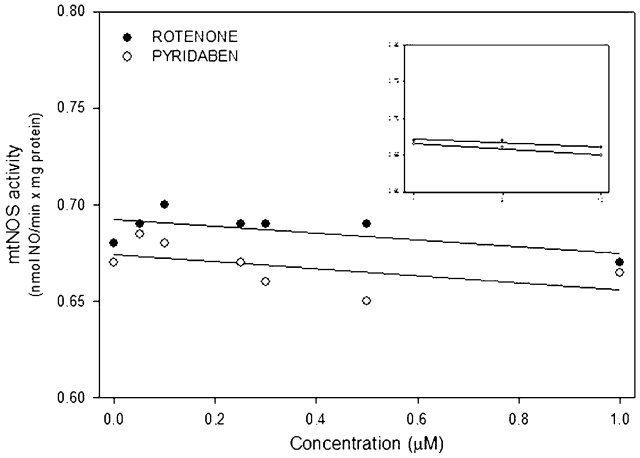

Fig. 5 Effects of rotenone and pyridaben on the biochemical mtNOS activity of mitochondrial membranes

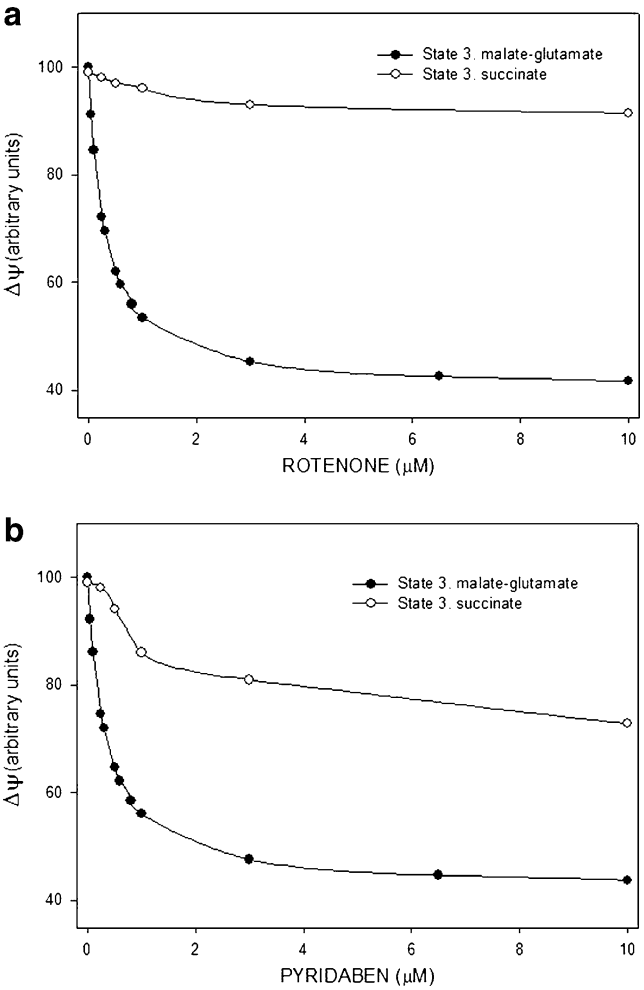

Fig. 6 Effects of rotenone and pyridaben on mitochondrial membrane potential as determined by rhodamine 123 (Rh123) accumulation and expressed as percentage of intramitochondrial Rh123 in state 3

in mitochondria from yeast (Schagger and Pfeiffer 2000; Cruciat et al. 2000; Krause et al. 2004), higher plants (Eubel et al. 2003; Eubel et al. 2004; Dudkina et al. 2005) and mammals (Schagger and Pfeiffer 2000; Schagger and Pfeiffer 2001; Schagger et al. 2004). Schäfer et al. 2006 reported the electron microscopic characterization of two respiratory chain supercomplexes: I_1III_2 and $I_1III_2IV_1$ in bovine heart mitochondria. The current concept is that the constitutive proteins of the inner mitochondrial membranes are densely packed with strong protein-protein interactions between respiratory complexes, F_1-ATP-synthase and regulatory proteins. Persichini et al. 2005, using electron microscopy, immunolocalization and co-precipitation showed a structural association between mtNOS and cytochrome oxidase at the C-terminal end of the Va subunit of cytochrome oxidase. Moreover, Poderoso's group extended the observation by using immuno-coprecipitation and reported that mtNOS is structurally adjacent to both complex I and cytochrome oxidase (Franco et al. 2006). This non-covalent bonding and interaction between mtNOS and complex I and complex IV is illustrated in Fig. 7.

It has been reported that NADH binding to complex I is accompanied by conformational changes in the hydrophilic subunits containing the flavin and the iron-sulfur centers (Belogrudov and Hatefi 1994). It is then likely that conformational changes are associated to electron transfer in complex I and that they affect the activity of adjacent mtNOS. This study claims that mtNOS functional activity, as production of NO and inhibition of O_2 uptake, are down regulated by complex I inhibitors beyond the direct effect

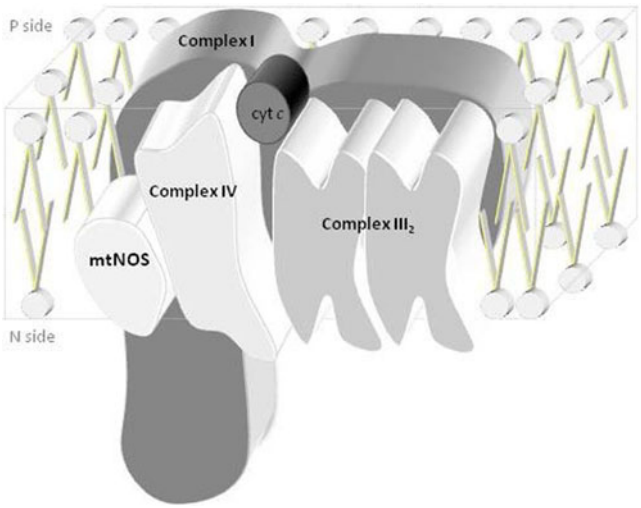

Fig. 7 Solid state model of the respiratory chain in the mitochondrial inner membrane showing mtNOS adjacent to both complex I and complex IV. The structural interaction between complex I and mtNOS is the molecular link between substrate availability (NADH; recognized by a conformational change at complex I) and the regulation of cytochrome oxidase activity by NO. Modified from ref. 44 (Navarro and Boveris 2008)

on complex I electron transfer. Our kinetic approach using complex I inhibitors and Ki values indicates two conformations of complex I, (a) one with a close interaction complex I-mtNOS and with relatively low Ki values for the inhibitors, 0.70–0.74 nmol/mg protein, and the other (b) with a looser interaction complex I-mtNOS and with higher Ki values, 2.6–3.0 nmol/mg protein. This point of view coincides with the recent observations of Parihar et al., who determined O_2 uptake and NO production in mitochondrial membranes and concluded that brain and liver mtNOS are functionally associated with complex I. When complex I is activated, mtNOS shows considerable enzymatic activity and generates NO, whereas inhibition of complex I by 5 μM rotenone leads mtNOS to loose its NO producing activity and to become a O_2^- source (Parihar et al. 2008a; Parihar et al. 2008b).

The functional association of mtNOS with complex I is bound to have implications in the slow and long development of neurodegenerative diseases. The so-called "complex I syndrome" describes complex I dysfunction as an inhibition in complex I activity, an increase in oxidation and nitration products and an increase in O_2^- and H_2O_2 production. This syndrome is classically associated with brain mitochondria in Parkinson's disease and in a series of other neuropathological situations (Cooper et al. 1992; Navarro and Boveris 2007; Navarro et al. 2009).

Before closing this study, it is convenient to consider the current knowledge on mtNOS. The production of NO by rat liver mitochondria was reported by Ghafourifar and Richter in 1997 (Ghafourifar and Richter 1997) and by Giulivi et al. in 1998 (Giulivi et al. 1998a) who used well controlled cell fractionation procedures. For a few years there was a skeptical attitude towards this enzyme, since the understanding at that time was dominated by the concepts of three genomic NOS (nNOS, iNOS and eNOS) and of "constitutive and Ca^{2+}-dependent" (nNOS and eNOS) or "inducible and Ca^{2+}-independent" (iNOS) activities (Ignarro 2000), with the problem of mtNOS being (at that time) reactive to anti-iNOS antibodies and Ca^{2+}-dependent. However, after a few years, mtNOS activity was reported in mitochondria from a series of rat and mouse organs and the idea of contamination faded away by considering unreasonable that very different tissues could produce a similar cytosolic contamination (Navarro and Boveris 2008). Giulivi and co-workers in a milestone contribution sequenced rat liver mtNOS fragments and identified the enzyme as an inner membrane integral protein of 130 kDa and as the transcript of nNOS, splice variant α, myristylated and phosphorylated (Elfering et al. 2002). Decisive evidence was provided by Kanai *et al.* with the electrochemical determination of the Ca^{2+}-induced NO release from a single mouse heart mitochondrion, a process that was abolished in nNOS -/- knock out mice (Kanai et al. 2004) and by Finocchietto *et al.* that reported increased mtNOS expression and activity by direct electroporation of nNOS rat gastrocnemius muscle (Finocchietto et al. 2008).

It is worth mentioning that a few years ago some authors were not able to measure mtNOS activity in mitochondria isolated from rats or mice (Brookes 2004; Tay et al. 2004). This absence of evidence is not evidence of absence, and it is likely explained by the no utilization of the sensitive spectrophotometric assay for NO detection in mitochondrial membranes (Boveris et al. 2002). The same assay for NO, but using the γ band of HbO_2, identified NO as the endothelium-derived relaxing factor (Ignarro et al. 1987). The use of the HbO_2 α band (577–591 nm) is an adaptation to the high light scattering of mitochondrial suspensions (Boveris et al. 2002).

The higher electrical potentials of state 4 ($\Delta\Psi$=170 mV) drive to higher mtNOS activities and the slightly lower potential of state 3 ($\Delta\Psi$=155–160 mV) leads to an about 50% lower mtNOS activity (Boveris et al. 2006; Valdez et al. 2006). In a clear demonstration of the voltage-regulation of mtNOS activity in isolated cells, Dedkova and Blatter reported a decreased cellular NO production by collapsing mitochondrial membrane potential with FCCP and co-localized NO and O_2^-/H_2O_2 in mitochondria (Dedkova et al. 2004; Dedkova and Blatter 2008). In short, mtNOS is an inner mitochondrial membrane enzyme with the property of a voltage-regulated enzyme.

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